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SUSTAINED RELEASE ANTIBIOTIC MICROSPHERES FOR ERADICATION OF BACTERIAL BIOFILM

BY

GODFRIED OWUSU-ABABIO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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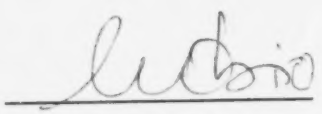

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MY WIFE, CHILDREN, PARENTS, FATHER AND MOTHER IN-LAW

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ABSTRACT

The decreased rate of killing of biofilm cells of *P. aeruginosa* growing on peritoneal implant device compared to planktonic cells under a condition mimicking *in vivo* situation indicates the requirement of higher sustained antibiotic concentration to eradicate bacterial biofilm. Cephalexin monohydrate (CPX) and ciprofloxacin hydrochloride (CFX) were formulated as sustained release poly(L-lactic acid) (PLA) microspheres using a coacervation technique by non-solvent addition, and characterized. Microspheres prepared with PLA 300,000 M.W. showed the least tendency to aggregate and were irregularly shaped compared to those prepared with either 50,000 or 100,000 M.W. The encapsulation efficiencies were >90% in all cases. The release of CPX or CFX at 37°C., in pH 7.4 phosphate buffer followed second order dissolution model kinetics for multiparticulate systems, and SEM pictures indicate a highly porous sponge-like external morphology. An optimum slowing down of drug release was observed when CPX microspheres were prepared at a stirring rate of 400 rpm. An increase in drug loading, a decrease in particle size or PLA M.W. resulted in an increase in the apparent second order release rate constant, k_2. The release of CPX (solubility = 40 mg/ml; pH 7.4) was faster than CFX (solubility = 5 mg/ml) from microspheres, and was sustained for a shorter period of time in the modified open chemostat system compared to the immediate decrease of antibiotic from peak concentrations. The concentration profiles were influenced by drug loading, microparticle size, and the dilution rate in the chemostat. This provided useful information about the optimum

dose to use for *in vivo* administration.

The effectiveness of CFX microspheres in eradicating planktonic and biofilm cells of *P. aeruginosa* and *S. aureus* was established in the open chemostat system. Microspheres of higher drug loading (71% w/w) provided adequate CFX concentration in the chemostat to eradicate biofilm of either bacterium and were characterized by lower maximum concentration (C_{max}), longer duration of C_{max}, and a higher concentration at the end of each 24 hour administration. The application of free CFX giving an equivalent C_{max} did not kill the bacteria because it was not sustained at levels above the minimum inhibitory concentration (MIC). The relative effectiveness of PLA microspheres of CFX against peritoneal implanted biofilm of *P. aeruginosa* was investigated in the rabbit model. Only rabbits receiving CFX (71% w/w) microspheres recovered from biofilm infection. Dialysate and serum concentrations after 12 hours (C_{12h}) were greater, and *P. aeruginosa* cell counts in the peritoneum were markedly reduced or eliminated as confirmed in scanning electron micrographs.

LIST OF ABBREVIATIONS

AUC	Area under concentration versus time curve
CAPD	Continuous ambulatory peritoneal dialysis
C_{12h}	Concentration at 12 h
C_{24}	Concentration at 24 h
CFU	Colony forming unit
CFX	Ciprofloxacin hydrochloride
C_{max}	Maximum concentration
CPX	Cephalexin monohydrate
DSC	Differential scanning calorimetry
EM	Electron micrograph
HPLC	High performance liquid chromatography
IDR	Initial dissolution rate
MIC	Minimum inhibitory concentration
M.W.	Molecular weight
NZW	New Zealand White
PBS	Phosphate buffer solution
PGA	Polyglycolic acid
PLA	Poly(L-lactic acid)
SEM	Scanning electron micrograph
T_g	Glass transition temperature
T_{max}	Time to reach maximum concentration
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.2 STATEMENT OF THE PROBLEM

Medical or prosthetic devices have been implanted in millions of human subjects to save lives or to improve the quality of life, and the application may be both short and long term (1-3). The implantation of several types of medical devices, such as aortic interposition grafts, perotoneovenous shunts, ventriculoperitoneal shunts, t-tubes or peritoneal dialysis catheters often result in infections (3), usually due to bacterial biofilm. Device-associated bacterial biofilm infections occur with either gram-positive or gram-negative bacteria and are of great concern to health-care professionals (3-7). Although most implant-related infections are caused by gram-positive bacteria, infections due to gram-negative bacteria are potentially more serious (3). The establishment of biofilm on the surfaces of medical devices is a means of survival for the pathogen and whose biofilm become more resistant to host immune defences and antibiotics with aging (4, 9-11). The current mode of treatment of biofilm-related infections involves frequent administration of high doses of antibiotics by conventional routes. In the case of catheter-related biofilm infections of the urinary tract, administration of antibiotics which are excreted primarily through the urine (e.g. ofloxacin) to which the causative organism is sensitive, appears to be very effective. This, however, is not the case with peritoneal catheters which are used to dialyze patients with chronic renal failure, especially the continuous ambulatory peritoneal dialysis (CAPD) patient. CAPD has become increasingly popular because it is less expensive and more convenient to the patient compared to hemodialysis. The patient is free to move about and

perform normal duties. CAPD has become a first-choice initial treatment for many patients, however peritonitis, particularly recurrent peritonitis, is a serious complication (12,13). There are several potential ports of entry for microbes leading to peritonitis but the major ones are intraluminal, periluminal, transmural, and vaginal leak infections (13). The most popular approach to treating peritoneal catheter-related biofilm infections involves the administration of antibiotics in peritoneal dialysate solution (14-18). The elimination half-life of most antibiotics in the peritoneum is relatively short due to rapid transperitoneal absorption. In peritonitis, the peritoneal membrane has an increased permeability and the half-life of the antibiotic in the peritoneum is shortened (14,18). Even though intraperitoneal administration of antibiotics appears to be effective in some patients, the emergence of resistant strains and recurrent peritonitis continue to be unsolved problems in many patients because of sub-therapeutic concentrations of antibiotic in the peritoneum near the end of the dwell time (15-17).

Various preventive solutions to medical device-infection related problems have been suggested but none appears to be very effective. Nickel et al. (19) reported that the incorporation of antibiotic in the wall of the outlet tube of a newly-developed catheter drainage system prevented colonization in some rabbits and delayed colonization in others for 8 days. Colonization occured after a period of time because the device became exhausted of antibiotic. The incorporation of nonsteroidal anti-inflammatory agents in medical polymers to reduce adherence of *Staphylococcus epidermidis* to medical polymers for 24 h was reported by

Farber et al. (20). This closed system *in vitro* test reduced adherence significantly in the presence of high concentrations of indomethacin over a 24 h period. However, it has not yet been shown to be effective *in vivo*.

In the absence of any effective preventive measure to control biofilm infections in patients undergoing CAPD, improvements in the current mode of treatment with antibiotics appear to be possible by applying a sustained release formulation.

CHAPTER 2

BACKGROUND

2.1 PERITONEAL DIALYSIS

Peritoneal dialysis probably represents solute and fluid exchange mainly between peritoneal capillary blood and dialysis solution in the peritoneal cavity (21). The peritoneal dialysis system can be considered as nature's version of a capillary kidney (22). For patients with end-stage renal disease, peritoneal dialysis has been shown to be a practical, safe, effective, and cost-effective alternative to chronic hemodialysis, and since 1985 peritoneal dialysis has been recognized as a major form of therapy for chronic renal failure. Peritonitis associated with this procedure is a limiting factor. There are two forms of peritoneal dialysis depending on how the technique is carried out, namely, intermittent peritoneal dialysis (IPD) and continuous ambulatory peritoneal dialysis (CAPD). IPD became unpopular because of inadequate dialysis clearance and dropouts to hemodialysis or CAPD.

2.1.1 Continuous Ambulatory Peritoneal Dialysis (CAPD)

The modern era of continuous ambulatory peritoneal dialysis can be traced to the work of Popovich and coworkers (23). In 1976 they described the intraperitoneal infusion of a patient with 2 litres of dialysate fluid. The dialysate was allowed to equilibrate for 5 h while the patient carried on with normal activities. The dialysate was then drained and fresh fluid was re-instilled again. Five exchanges per day, 7 days per week were carried out. Later, experience with more patients was reported by Popovich et al. (1978), and the name of the method was changed to CAPD (24).

Today, peritoneal dialysis encompasses a closed system of commercially

prepared dialysate fluid packaged in a plastic bag that is connected by Silastic tubing to the Tenckhoff catheter. The effectiveness of CAPD is achieved by hyperosmolar ultrafiltration across the peritoneal membrane. Usually 1 to 2 litres of dialysate is instilled for a dwell time of 4 to 8 h, and cycles are repeated every 6 h. Empty bags are reused for recovery of effluent drainage by gravity at the end of a cycle.

CAPD has certain advantages over hemodialysis or IPD. The blood chemistries of patients on CAPD are steady after a few weeks of treatment because there is a constant removal of waste product from the body. This makes CAPD the most physiologic effective way of dialysis. Because of sufficient fluid removal each day, the fluid allowance of the patients is more liberal. The blood pressure is usually well-controlled. CAPD patients show a rise in hematocrit which is not seen in other dialysis methods. The clinical condition of the patient is good provided that complications like peritonitis do not occur and the patient can eat sufficient amount of protein to compensate for the protein loss with dialysis.

Special indications are: dialysis of diabetic patients because of easier blood sugar control (usually insulin is administered intraperitoneally) and dialysis of children.

2.2 PERITONITIS

The most complete definition of peritonitis in patients treated with CAPD was given by Vas (25) who proposed that this complication should be diagnosed if any two of the following three criteria were present: 1) abdominal pain and/or

tenderness; 2) cloudy dialysate with more than 100 neutrophils/mm^3; 3) micro-organisms in the peritoneal fluid. More than half of all peritonitis episodes are observed in only 25% of all patients on CAPD (26,27). Approximately 60% of patients on CAPD will have developed at least one episode of peritonitis during the first year of dialysis (27,28). Infectious peritonitis during CAPD results mainly from contamination of the peritoneal cavity by bacteria, and with lesser frequency, by fungi. The majority of peritonitis episodes are due to gram-positive bacteria which are found in 55-70% of isolates (25,29,30). *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Streptococcus* species are the three bacteria most often encountered. *Staphylococcus epidermidis* is responsible for about 40% of peritonitis. The two major groups of gram-negative bacteria responsible for 15-20% of peritonitis are the *Enterobacteriaceae* and *Pseudomonas* species. Fungi are recognized today as an etiologic factor in 5% of peritonitis (25,31).

2.2.1 Consequences of Peritonitis

Although peritonitis in CAPD patients usually follows a benign course, this complication is potentially lethal, particularly in the elderly and in patients with associated diseases (e.g. cardiovascular atherosclerosis, diabetes mellitus, malnutrition) (32). Peritoneal infection was shown to enhance urea and creatinine peritoneal clearances, to increase glucose absorption and to decrease the volume of dialysate effluent. Impaired ultrafiltration is due to the rapid influx of glucose into the blood stream and to a parallel fall in osmotic gradient. Experimental data suggest that changes in permeability predominate as a consequence of histamine

release and complement activation with formation of C_{5a} anaphylatoxin (33). Peritonitis is a cause of negative nitrogen balance provoked by decreased protein intake, accelerated tissue breakdown and raised protein losses (34). Pulmonary complications are facilitated by both upward displacement due to bowel distension and by pulmonary edema induced by inadequate fluid removal (32,35). Peritoneal inflammation is associated with fibrinogen-rich exudate and depressed fibrinolytic activity (35). As a result, fibrin clots are frequent cause of impaired drainage, and fibrin strands result in adhesion formation. Repeated and/or severe peritoneal infection may be one of the major pathogenetic mechanisms of sclerosing encapsulating peritonitis (36).

2.2.2 Mortality

Even though CAPD peritonitis is considered to be a rather benign illness, it is reported as a cause of death in 2.4 - 12.2% of patients receiving CAPD (32,37), and represents 17.2 - 50% of all deaths (37,38). The main cause of death is generalized sepsis with septic shock. Other causes include uncontrolled peritoneal infection, septicemia, pulmonary infection, acute myocardial infarction, and sclerosing encapsulating peritonitis (32).

2.2.3 Recurrent Peritonitis

The primary complication which limits the applicability of CAPD to patients with end-stage renal disease is recurrent peritonitis. Relapse or recurrent peritonitis has been noted after 10-30% of peritonitis episodes (29,39). Such episodes are mainly due to treatment failure and thought to be due to extracellular slime substance or

biofilm produced by these organisms which serve to protect them from host defences as well as from antimicrobial action (40-42). Frequent recurrent peritonitis or relapse with the same organism results in the removal of catheter and placement of the patient on hemodialysis because continuation of CAPD may become a hazard to the patient.

2.3 BIOFILM

The term "biofilm" refers to microbes that are able to attach firmly to solid surfaces, such as catheters and other prosthetic devices and produce exopolysaccharide (glycocalyx) matrix, inside of which microcolonies develop. These microcolonies coalesce to form biofilm (4,5,43).

2.3.1 Physiology and Composition of Bacteria in Biofilm

The physiology of biofilm cells is extremely complex and is profoundly different from those grown planktonically (5,10,44). It is generally believed the attachment of microbes to solid surfaces can profoundly influence their metabolic activities in a way that is not easily predicted from current knowledge (5,44,45). The restriction of certain available essential nutrients is known to influence the physiology of bacterial pathogens which in turn can profoundly affect their susceptibilities to antibiotics (5,10,46). For example, the cultivation of *Pseudomonas aeruginosa* under conditions of magnesium depletion has been shown to result in an increase in the resistance of the organism to polymyxin B, EDTA and aminoglycosides (47-49). Biofilms are typically composed of cells embedded at various levels in a highly

hydrated, polyanionic matrix (5). Observation of living biofilms by the scanning confocal laser microscopic technique has established that biofilms produced by all bacterial species examined to date have similar structures and that cells typically occupy 5 to 35% of the thickness within these adherent structures (50,51).

2.3.2 Resistance of Bacteria in Biofilm to Antibiotics

Anwar et al. proposed a model for describing the complex nature of the resistance of bacteria in biofilm to antibiotics (11). Surface biofilm cells resemble planktonic cells, being large with cell membranes permeable to nutrients and antibiotics. Upon exposure to antibiotic the number of antibiotic molecules penetrating these cells is likely to be greater than that needed to inactivate the cells. These excess antibiotic molecules not engaged in cell inactivation are probably destroyed by antibiotic-degrading enzymes, or are involved in non-specific interaction with other cellular components. Such behavior significantly reduces the number of antibiotic molecules available to kill the biofilm cells deeply embeded in the thick glycocalyx matrix. The glycocalyx produced by biofilm cells is negatively-charged and is known to function like an ion-exchange resin capable of binding a very large number of antibiotic molecules, thereby preventing them from reaching the deeply embeded biofilm cells (50,51). Antibiotic degrading enzymes, such as beta lactamase, may also be immobilized on the glycocalyx matrix, thus incoming antibiotic (beta-lactam) molecules are inactivated. Also, embedded biofilm cells are not generally engaged in active cell division and are smaller in size than planktonic or surface biofilm cells. Slow growing cells are generally less susceptible to

antibiotics, presumably because the membranes of these cells are less permeable. In view of such mechanisms, embedded biofilm cells may have sufficient time to express antibiotic-resistant factors such as antibiotic-degrading ezymes (52). In addition these slow-growing biofilm cells become altered in some way making them less vulnerable to attack by antibiotics.

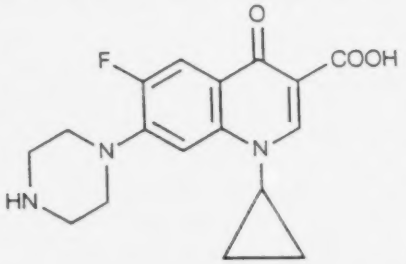
A high concentration of antibiotic, referred to as biofilm eradication concentration (BEC) is required for the eradication of bacteria growing in glycocalyx-protected biofilm. Normally, the BEC of antibiotic is very difficult to achieve in the body due to the processes of distribution and elimination.

2.4 ANTIBIOTICS

Ciprofloxacin and cephalexin have both been investigated for their activities against biofilm and have been administered to patients by the oral and peritoneal routes but inadequate drug concentrations to eradicate bacterial biofilm were obtained (15-17,29).

2.4.1 Ciprofloxacin

Ciprofloxacin is a fluoroquinolone antibiotic with activity against both gram-positive and gram-negative bacteria *in vitro* (53). The structural formula of ciprofloxacin is:



1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carbonic acid.

The isoelectric point is at pH 7.4. The 6-fluoro substituent is mainly responsible for its potent antibacterial activity and the cyclopropyl substituent also contributes to this. The 7-piperazino group constitutes the anti-pseudomonal activity.

2.4.1.1 Mechanism of Action

The mechanism of action of ciprofloxacin and other quinolones is complex and not fully elucidated. Ciprofloxacin is an inhibitor of DNA gyrase, an essential component of the DNA replication system. DNA gyrase introduces about 333 negative supercoilings into each domain. Ciprofloxacin therefore acts by preventing DNA from introducing this negative spherical twist into its strands and, consequently, increasing its spacial requirement within the bacterial cell. Thus, the cell can no longer replicate (54,55). DNA gyrase is composed of 4 subunits, 2 alpha and 2 beta. The alpha subunits are the target of ciprofloxacin and other quinolones but mutations that affect the beta subunits change bacterial sensitivity to 4-quinolones. Hence, the 4-quinolones would seem to affect both beta and alpha subunits of DNA gyrase (55,56). Inhibition of purified DNA gyrase has correlated with antibacterial activity (56,57). It is speculated that ciprofloxacin and ofloxacin may also act by an additional killing mechanism that increases their potencies, which may explain lower frequencies of mutants resistant to these agents compared with other quinolones (55).

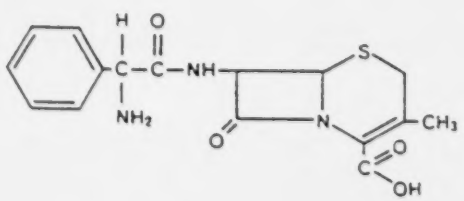
2.4.1.2 Antimicrobial Spectrum

Ciprofloxacin is bactericidal against *Staphylococcus* and *Streptococci* species including methicillin-resistant *Staphylococcus aureus* (58-60). It is also active

against *Hemophilus influenzae*, *Escherichia coli*, and *Enterobacter* sp. Ciprofloxacin is the most effective quinolone against *Pseudomonas aeruginosa*. *Neisseria gonorrhoeae*, including penicillin-resistant strains, are very sensitive to fluoroquinolones, especially ciprofloxacin. Other orgainisms that are sensitive to ciprofloxacin include *Chlamydia trachomatis*, *Gardnerella vaginalis*, *Salmonella* sp., *Shigella* sp., *Campylobacter jejuni*, *Mycobacterium* sp. and *Legionella* sp. The broad spectrum and high bactericidal activity of ciprofloxacin make it a suitable candidate for the treatment of device-associated biofilm infections.

2.4.2 Cephalexin

Cephalexin is a cephalosporin antibiotic which is active against both gram-positive and gram-negative bacteria *in vitro* (61). The structural formula of cephalexin is:



7-(2-amino-2-phenyl-acetamido)-3-methyl-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid. The isoelectric point is at pH 4.5.

2.4.2.1 Mechanism of Action

The bactericidal action of cephalexin depends on its ability to reach and bind to penicillin-binding proteins located in bacterial cytoplasmic membranes. Cephalexin

inhibits bacterial septum and cell wall synthesis, probably by acylation of membrane-bound transpeptidase enzymes. This prevents cross-linkage of peptidoglycan chains which are necessary for bacterial cell wall strength and rigidity. Also, cell division and growth are inhibited and lysis and elongation of susceptible bacteria frequently occur. Rapidly dividing bacteria are the most susceptible to the action of cephalosporins.

2.4.2.2 Antimicrobial Spectrum

Cephalexin is active against most gram-positive bacteria, including beta lactamase producing *Staphylococcus aureus* (62) and most *Streptococci.* Pneumococci and *Neisseria gonorrhoeae* are inhibited by concentrations that are achieved in the serum. Gram-negative bacteria activity is generally limited to *Escherichia coli, Klebsiella* and *Proteus mirabilis* (62). Cephalexin is active against bacterial biofilm when administered in conjunction with an aminoglycoside or a fluoroquinolone.

The interaction of antibiotics with glycocalyx of bacterial biofilm varies depending on ionization of the antibiotic at the pH which the biofilm is growing. At pH 7.4, the cephalexin molecule has a net negative charge (isoelectric point = pH 4.5) and could be repelled by the negatively charged glycocalyx, which would indicate low activity against bacteria in biofilm. Ciprofloxacin molecule has no net charge at this pH (isoelectric point = pH 7.4) which would indicate that the drug could penetrate the negatively glycocalyx.

2.5 FORMULATION AND CHARACTERIZATION OF POLYMERIC ANTIBIOTIC MICROPARTICLES

Microcapsules developed and used in medicine consist of a solid or liquid core material containing one or more drugs enclosed in a polymeric coating (63). When no distinct coating and core regions are distinguishable, the analogous term used is microsphere. Depending on the manufacturing process, various types of microcapsule structures can be obtained and, as a result, there is considerable confusion in the nomenclature of the resulting microparticles (64,65). The most common type of microcapsule is the mononuclear spherical type (Figure 2.1). Microcapsules have particle sizes in the range of 1 to 2000 μm.

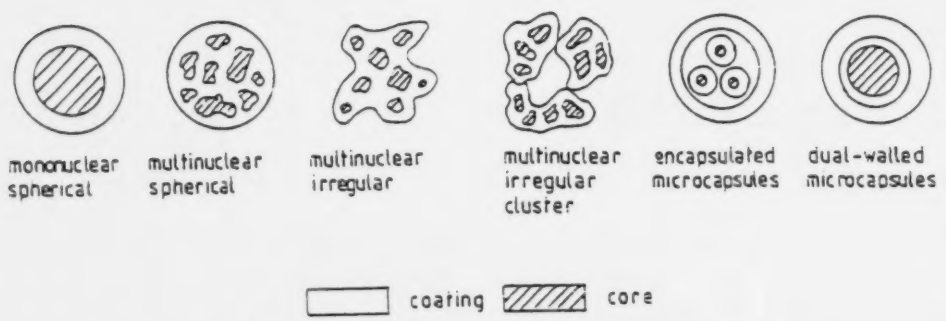


Figure 2.1 Some typical structures of microcapsules (taken from Deasy, P. B., Ref. 63)

Drugs have been prepared as microcapsules:

1. as an aid to handling or storage (66);
2. to disguise the unpleasant taste of a number of drugs (63);
3. to reduce gastric and other gastrointestinal tract irritation (67); and
4. to provide sustained-release medication (68,69).

In general, the goal of a sustained release dosage form is to maintain therapeutic blood, tissue or localized levels of the drug for an extended period of time. This is generally accomplished by attempting to obtain " zero-order" release from the dosage form. Zero-order release (i.e., constant release) constitutes drug release from the microcapsule which is independent of the amount of drug in the microcapsule. Many sustained-release systems do not attain this type of release but mimic zero-order release by providing drug in a slow first-order fashion (i.e., concentration dependent). Sustained-release delivery forms tend to reduce side effects by maintaining the plasma or localized drug levels only within the therapeutic range for a prolonged pariod of time.

2.5.1 Polymeric Coating Materials

Selection of the coating polymer is based upon a number of criteria (70). It is important that the polymer have an appropriate diffusivity, i.e., the permeability of the polymer to the drug must yield a therapeutically functional drug release rate. In addition, the polymer must be non-toxic and, if biodegradable, it must yield non-toxic products. If the delivery system is to be implanted in the body, the polymer must also be biocompatible with the surrounding tissues. The polymer should be inexpensive and possess good mechanical properties to allow easy fabrication and manufacture. It is preferred that the polymer has already been approved by the FDA for use in dosage forms. A predetermined release rate of drug can be obtained by suitable choice of polymer and an adequate design of the system.

Polymers that have been studied for drug delivery application can be classified

into four categories depending on their physicochemical properties.

A. Nonbioerodible hydrophobic polymers:- are generally inert, e.g., ethylene vinylacetate copolymer (EVA), polydimethyl siloxane (PDS), polyether urethane (PEU), polyvinyl chloride (PVC), polyethylene, and ethylcellulose.

B. Hydrogels:- are cross-linked polymers that swell but do not dissolve in water, e.g., polyhydroxyethyl methacrylate (PHEMA), polyvinyl alcohol (PVA), polyethylene oxide (PEO), and cross-linked polyvinyl pyrrolidone (PVP-XC).

C. Bioerodible polymers:- slowly disappear from the site of administration due to a chemical reaction such as hydrolysis, e.g. polylactic acid (PLLA), polyglycolic acid (PGA), PLLA/PGA copolymers, polycaprolactone (PCL), polyhydroxybutyrate (PHB), polyamino acids, labile polyesters and several generic classes, such as poly(orthoesters) and polyanhydrides.

D. Water soluble polymers:- such as pH-dependent cellulose acetate phthalate (CAP), hydroxypropyl methylcellulose (HPMC), hydroxypropylcellulose (HPC), hydroxyethyl cellulose (HEC), and carboxymethylcellulose (CMC).

2.5.1.1 Poly(lactic acid) and Poly(glycolic acid)

Among the bioerodible polymers, poly(lactic acid) (PLLA) and poly(glycolic acid) (PGA) are the most widely used in medicine. They elicit minimal inflammatory responses, and degrade to products which are normal metabolites of carbohydrate metabolism and are therefore, eventually absorbed without any accumulation in the vital organs (71-75). PGA and poly(L-lactic acid) (PLA) have been used as

absorbable sutures (76,77) and as vascular prostheses (78). The two monomers i.e., lactic and glycolic acids can be copolymerized to prepare a series of related random copolymers (79). PLLA, PLA or PGA, and copolymers of these have been used for the microencapsulation of several drugs for sustained drug delivery (80-82).

Differential scanning calorimetry (DSC) is particularly important in studying the morphology of the glycolic acid/lactic acid family of polymers. PGA, PLLA, and PLA show glass transitions (T_g) of 37, 67, and 57°C, respectively. On the other hand, poly(glycolic acid-co-DL-lactic acid), a copolymer, has a T_g of 45°C (79,83). X-ray and DSC have shown that PGA has a typical crystallinity of approximately 50% whereas PLA is approximately 37%. Copolymers of these are amorphous between 25 and 70 m% glycolic acid (79). Poly-dl-lactic acid (PLLA) is also amorphous.

The family of polyglycolic/polylactic acids undergoes hydrolytic degradation of the ester bonds. Factors that influence the rate of degradation of these polymers are molecular weight, surface quality, and composition of the polymer. PLA degrades within months or years whereas PLLA degrades within weeks or months (71,75,83,84). The physical properties of some of these polymers commercially available from Medisorb, a Stolie-Dupont company are shown in Table 2.1.

2.5.2 Microencapsulation Techniques

A great many microencapsulation techniques are available and new ones are being developed each year. A number of authors have classified the various microencapsulation techniques (64,85-88), but in this thesis the classification

Table 2.1. Physical properties of PGA, PLA, PLLA and copolymers containing 85 or 50% PLLA synthesized by Medisorb.

Grade	Glass Transition Temperature (°C)	Melting Point (°C)	Inherent Viscosity (dl/g)	Break Stress (Kpsi)
PGA	45-50	225-235	1.2	11.2
PLA	40-45	NA	0.6	6.6
PLLA	55-60	170-175	0.9	8.3
8515 (PLLA/PGA)	40-45	NA	0.6	6.3
5050 (PLLA/PGA)	45-50	NA	0.6	7.7

The average molecular weight of PGA, and PLA is around 100,000 while that of PLLA and the copolymers is 40,000 - 100,000. NA means information not available.

based on the starting material is adopted. This is shown in Table 2.2. The major microencapsulation techniques include coacervation (phase separation procedures), spray-drying, interfacial polycondensation, pan coating, and air suspension coating (66,89,90). Of all the techniques, coacervation is the most widely used (90).

2.5.2.1 Coacervation or Phase Separation Procedures

The term "coacervation" has been used to describe the salting out or phase separation of lyophilic sols as liquid droplets. In the microencapsulation process,

Table 2.2. General microencapsulation techniques

From Polymeric Starting Materials	From Monomeric Starting Materials
Coacervation/phase separation	Interfacial polymerization
Pan coating	In-situ polymerization
Fluidized bed coating/air suspension coating	Emulsion polymerization
Spray-drying	Suspension polymerization
Suspension crosslinking	

the wall-forming polymer in a solution containing suspended core material is induced to separate as a liquid phase by creating nonsolvent conditions for the polymer, by decreasing the temperature, adding a phase inducer, or adding another polymer that has higher solubility in the solvent. When the wall-forming polymer separates as a polymer-rich liquid phase, this phase is called a coacervate and the process is called simple coacervation. The coacervate must adhere and wet the suspended core particles or droplets and coalesce to form a continuous coating. The other type of coacervation referred to as complex coacervation, depends on the pH and involves another colloid. The coacervate forms due to complementary charge neutralization (e.g. gelatin and acacia) by pH

adjustment (66,89,90).

The solvent evaporation process is yet another means of achieving phase separation which usually results in the production of matrix particles or microspheres. Reduced pressure and/or applied heat is required to evaporate the organic solvent under controlled conditions to yield the product (66).

2.5.2.2 Spray-Drying

This sometimes serves as a microencapsulation technique when the active material dissolved or suspended in a melt or polymer solution is atomized in a drying chamber in a spray dryer forming small particles of the drug and polymer intimately mixed (66,90). The main advantage of this technique is the ability to handle labile materials because of the short contact time in the drying chamber. However, the coating produced by spray-drying tends to be porous which is satisfactory for taste-masking and some other purposes but not for controlled release (66).

The ease of deposition of a uniform coating onto the surfaces of solid drug particles depends on the shape and size distribution of the particles. It is much easier to deposit uniform coatings on regular spherical particles of narrow size range devoid of sharp edges. Spray-drying of dissolved particles in a solvent can be used to obtain spherical particles of narrow size distribution (91,92).

2.5.3 Experimental Means of Determining Release Kinetics from Microparticles

The characterization of drug release from sustained release formulations has

generally been carried out with an existing apparatus used for dissolution testing (93,94). The "Spin-Filter Stationary Basket" dissolution test apparatus developed by Shah et al. (95) is widely applied in formulation development and has been recommended for approval by the FDA. The spin-filter test apparatus has been found to provide a reliable and convenient means for determining *in vitro* dissolution parameters of tablets, capsules, powders, suspensions and most other solid dosage forms, including sustained release formulations (96). These methods are all of the closed-compartment type enabling release profiles from drug delivery systems to be characterized as either zero order, first order, matrix release or combinations of these.

Recently, a standardized flow-through cell method, official in the European Pharmacopoeia, has been suggested as an alternative to existing Pharmacopoeia dissolution testing methods. This method is representative of the open compartment model.

2.5.3.1 Kinetics of Drug Release from Polymeric Microparticles *In Vitro*

Generally, release of drug from dosage forms *in vitro* is controlled by the physicochemical properties of the drug and the nature of the delivery system. The release rate of a drug from a polymeric device is normally controlled by the device itself. Mathematical models used to describe the kinetics of drug release from microparticles pertain to microcapsules or release from a microsphere. Microparticles are often irregular in shape so that conventional models based on spherical, cylindrical, slab, or other regular geometries often show a poor fit of

release data (66).

The types of polymeric controlled release devices can be categorized as: i) those in which the active agents form a core surrounded by an inert diffusion barrier (i.e, reservoir devices); ii) those in which the active agent is dissolved or dispersed in an inert diffusion medium (i.e., monolithic devices); and iii) those in which the agent is bound to a polymer backbone producing a release rate controlled by the rate of hydrolysis of the bond.

2.5.3.2 Reservoir Devices

Drug encapsulated in reservoir devices are released by kinetics depending on the thermodynamic activity of the drug in the reservoir. If the thermodynamic activity of the drug is maintained constant within the core for a period of time, the transport of dissolved drug across the surrounding membrane will be constant, i.e. follow zero-order kinetics. On the other hand, if the thermodynamic activity of the drug is not maintained constant (i.e. the drug in the core is dissolved at a concentration below saturation) the release rate decreases exponentially, and follows first-order kinetics (97).

The release rate of drug from a reservoir device is also influenced by the diffusivity of the drug in the wall-forming polymer and the device configuration or geometry. For a spherical microcapsule with an inert homogeneous coating (Figure 2.2), the steady-state release rate of a moderately soluble drug diffusing through a polymer as derived from Ficks first law is given by:

$$\frac{dM_t}{dt} = 4\pi DK\Delta C \frac{r_o r_i}{r_o - r_i}$$

where dM_t/dt is the steady-state release rate at time t, DK is the polymer wall permeability, r_i is the internal radius, r_o is the external radius, and ΔC is the difference between the internal and external drug concentrations (97). In the limit as the ratio of external radius to internal radius approaches infinity ($r_o/r_i \rightarrow \infty$),

$$\frac{dM_t}{dt} = 4\pi DK r_i \Delta C$$

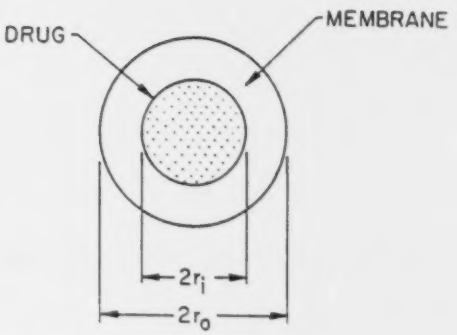


Figure 2.2 Idealized diagram of a cross section of a sphere. (taken from Baker and Lonsdale, Ref. 97)

i.e, the release rate becomes independent of outer radius of the device. Baker and Lonsdale (97) have stated that when r_o/r_i exceeds a value of about 4, further increase in device size for a fixed-core radius will not significantly change the release rate. This means that increasing the thickness of the coating on microcapsules beyond $r_o/r_i > 4$ will negatively influence the drug release rate, since it might delay the onset of the steady-state release.

In many cases, constant release of drug from reservoir devices is of short to medium duration depending on the water solubility of the drug, then the activity of drug in the core decreases with time as water enters and dilutes the drug solution. Under sink conditions, the amount of drug M_t remaining in the microcapsule at any time, t, is given by:

$$M_{1t}=M_{\infty}\exp\left[-\frac{ADKt}{\ell V_1}\right]$$

where M_∞ is the total mass of drug in the microcapsule initially, ℓ is the diffusional path length, V_1 is the microcapsule reservoir volume and A is the surface area of the microcapsule. The release rate is given by:

$$\frac{dM_{1t}}{dt}=-\frac{M_{\infty}ADK}{\ell V_1}\exp\left[-\frac{ADKt}{\ell V_1}\right]$$

The time required for the microcapsules to release half of the drug, $t_{½}$ is given by the expression:

$$t_{½}=0.693\frac{\ell V_1}{ADK}$$

2.5.3.3 Monolithic Devices (Dispersed Drug)

The fraction of drug released from a homogenous matrix of a slab in which drug is dispersed has been described by Higuchi (98):

$$M_t=A[DtC_s(2C_o-C_s)]^{½}$$ where $C_o >> C_s$,

$$M_t\approx A(2DtC_sC_o)^{½}$$

M_t is the amount of drug released after time t, D is the diffusion coefficient of the solute in solution, C_o is the total concentration of drug in the matrix, C_s is the solubility of the drug in the membrane. The release rate at any time is then given by:

$$\frac{dM_t}{dt} = \frac{A}{2}\left[\frac{DC_s}{t}(2C_o - C_s)\right]^{1/2} \qquad \text{where } C_o >> C_s,$$

$$\frac{dM_t}{dt} \approx \frac{A}{2}\left[\frac{2DC_sC_o}{t}\right]^{1/2}$$

where dM_t/dt is the steady-state release rate at time t.

The fraction of drug released from a homogenous matrix of spherical geometry in which drug is dispersed as a function of time has also been described by Higuchi (98), and expressed by Baker and Lonsdale (97) as:

$$\frac{M_t}{M_\infty} = \frac{3}{2}\left[1 - [1 - \frac{M_t}{M_\infty}]^{\frac{2}{3}}\right] - \frac{3DC_s}{r_o^2 C_o} \cdot t$$

The release rate at any time, t, during this period is given by:

$$\frac{dM_t}{dt} = M_\infty \frac{3C_sD}{r_o^2 C_o}\left[\frac{[1 - M_t/M_\infty]^{\frac{1}{3}}}{1 - [1 - M_t/M_\infty]^{\frac{1}{3}}}\right]$$

where M_∞ is the total amount of drug released after time ∞ (= mass of drug initially in the matrix), and r_o is the outside radius.

2.6 THE CHEMOSTAT SYSTEM

The chemostat was first described by Ombaka et al. (99), for continuous cultivation of planktonic cells under controlled conditions of nutrient limitation. Subsequently, Anwar et al. (99,101) modified this system and applied the chemostat for continuous cultivation of biofilm and planktonic cells under controlled conditions. The modified chemostat is an open system into which fresh medium is continuously pumped at a pre-determined rate and from which the contents are drained from the effluent port at the same rate.

2.6.1 Application of the Chemostat System in Biofilm Research

The open chemostat system has been used by Anwar et al. to study the biofilm and its susceptibility to antibiotics (100-103). In the chemostat, the organism is allowed to colonize on an inert solid surface and grow at a slow rate under conditions of iron restriction before addition of antibiotic. Alternatively, the colonized material in the chemostat can be removed and exposed to fixed concentrations of antibiotics in test tubes. Anwar et al. reported that young biofilm cells of *Pseudomonas aeruginosa* or *Staphylococcus aureus* were more sensitive to antibiotics than aged biofilm cells (9,100,101,103). Biofilm cells harvested on day 2 were described as young and those harvested on day 7 were described as old. *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been used extensively in research, and much is known about their nutrition (46-51), pathogenesis (2-4) and interaction with antibiotics (52-65) including their uptake (104). These represent bacteria commonly found in medical device related infections and their

biofilms are therefore suitable for testing against sustained release antibiotic formulations in the chemostat and in the rabbit peritoneal model.

2.7 THE RABBIT PERITONEAL MODEL

This model has been used to study the pathobiology of infectious biofilms on biomaterial surfaces (105) and the mechanism of persistent infection associated with peritoneal implants (106). The New Zealand white rabbit (about 3 - 3.5 kg) which is anaethesized with halothane, a midline abdominal incision is perfomed and a precolonized device of Silastic (Figure 2.3) is implanted in the peritoneal cavity. A Tenchoff catheter is placed in the peritoneal cavity so that its tip is located adjacent to the test implant and is used to inject or remove dialysis fluid. Antibiotic solution is injected into the peritoneum in the dialysis fluid and subsequently, samples of the residual amounts are removed via the catheter.

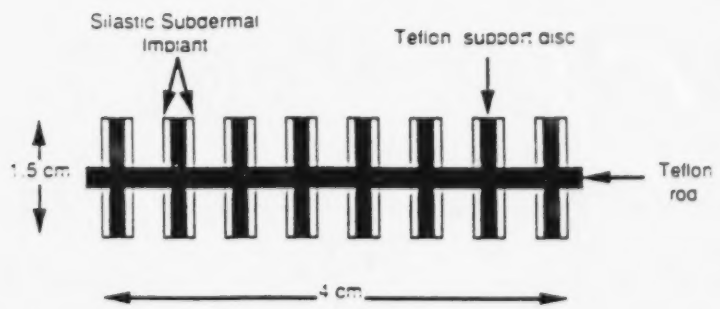


Figure 2.3 Diagramatic representation of the implant devices (taken from Ward et al. Ref. 106)

A. AIM

The aim of this research is to develop a microparticulate sustained release delivery system of antibiotic which eradicates bacterial biofilm *in vitro* in the open chemostat system and *in vivo* in the rabbit intraperitoneal model.

B. HYPOTHESIS

Prolonged exposure of device-associated bacterial biofilm at inhibitory concentrations of antibiotic in the peritoneum will completely eradicate biofilm and prevent re-current infections. A biodegradable polymeric microparticulate formulation injected intraperitoneally in dialysate can be designed for this purpose.

C. OBJECTIVES

1. To study the significance of antibiotic clearance rate on the eradication of planktonic and biofilm cells in the open chemostat.
2. To formulate and characterize sustained release polymeric antibiotic microparticles containing a beta-lactam antibiotic, represented by cephalexin, or a fluoroquinolone antibiotic represented by ciprofloxacin using biodegradable poly(L-lactic acid) or PLA/PGA copolymer as the rate-controlling polymer.
3. To evaluate the chemostat as a model to study the kinetics of release of antibiotic from sustained release microparticles.
4. To test the the effectiveness of sustained release polymeric microparticles of ciprofloxacin HCl *in vitro* to eradicate bacterial biofilms of *Pseudomonas*

aeruginosa and *Staphylococcus aureus* in the open chemostat.

5. To test the effectiveness of sustained release polymeric microparticles of ciprofloxacin HCl *in vivo* in the rabbit peritoneal model against *Pseudomonas aeruginosa* biofilm infections associated with a Silastic implant and peritoneal catheter.

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CHAPTER 3

FORMULATION AND RELEASE KINETICS OF CEPHALEXIN MONOHYDRATE FROM BIODEGRADABLE POLYMERIC MICROSPHERES

A version of this chapter has been submitted for publication to J. Microencapsulation

3.1 INTRODUCTION

Cephalexin is a first generation cephalosporin antibiotic with a high activity against a wide spectrum of gram-positive bacteria but only modest activity against gram-negative bacteria. Cephalexin is still being used clinically but like several other antibiotics administered parenterally or orally, therapeutic tissue levels of cephalexin at localized sites of infection beyond the systemic circulation are difficult to achieve without high concentrations and eliciting toxicities. Thus, when bacterial infections that occur in remote areas of the body, such as the peritoneum, are exposed to sub-therapeutic concentrations of cephalexin, bacterial resistance can occur. Administering the antibiotic I.P. to treat local infections associated with various devices, for example with an in-dwelling catheter, appears to be effective initially but recurrent infections occur, presumably because the antibiotic concentration in the peritioneal cavity fluids is rapidly decreased due to absorption and transport of drug to the systemic circulation (1-3). Alternatively, the effectiveness of the antibiotic may be improved if formulated as a sustained release product. A polymeric microparticulate drug delivery system could be formulated to achieve this objective for convenient administration of cephalexin. Many sustained release systems for I.M. injection or implantation are designed for long-term delivery of potent drugs. However, antibiotics administered I.P. can not be sustained for long periods of time without sacrificing effective concentration levels. Furthermore, when a patient is undergoing continuous ambulatory peritioneal dialysis (CAPD) the dwell-time of dialysate is only 6-8 h, at which time

any residual sustained release system is removed. One of the objectives, therefore, is to administer a delivery system that would be sustained but release most of the drug within this period of time.

Poly(lactic acid) has been widely used as a biodegradable polymer and is well-tolerated by patients (4-7). Various types of dosage forms, drugs, and preparation methods have been used with this polymer providing a variety of release profiles and kinetics (8-11). Also, its availability as different molecular weight polymers of known glass transition temperatures (T_g) provides opportunties in optimization of a drug delivery system. This study was undertaken to determine the characteristics of PLA microspheres of water-soluble cephalexin monohydrate and describe the kinetics of release as a function of several formulation factors.

3.2 EXPERIMENTAL

3.2.1 Materials

Poly (L-lactic acid), (50,000 and 100,000 M.W.) were obtained from Polysciences Inc. (Warrington, PA) and cephalexin monohydrate was obtained from Sigma Chemical Co., St. Louis, MO. Methylene chloride, diethyl ether and all other chemicals were reagent grade. Demineralized, double-distilled water was used.

3.2.2 Preparation of Microspheres

Raw cephalexin (i.e. not processed) or spray-dried cephalexin (Buchi 190 mini spray dryer, Brinkman Instruments, Toronto, Ont.) was microencapsulated by a

coacervation procedure involving phase separation of PLA from methylene chloride by the nonsolvent addition of diethyl ether. Typically, an amount of cephalexin was dispersed in methylene chloride by probe sonication (Heat Systems, Model W-375, Plainview, N.Y.). This was mixed with a solution of PLA (100,000 M.W., 400 mg) in methylene chloride to make a total volume of 10 ml, and magnetically stirred (Ikamag stir plate, 400 rpm). Diethyl ether (36 ml) was added dropwise at a constant rate of 0.2 ml/min using a syringe pump (Sage Instruments, Boston, MA) over a period of three hours. After formation, the microspheres were harvested by filtration, (Whatman No. 5, nominal porosity 2.5 μm), washed with 2 % polyethylene glycol 400 solution to reduce aggregation during harvesting, dried over calcium chloride under vacuum in a desiccator, then sieve-sized into various fractions (U.S. standard sieves). The effect of preparation conditions, such as the stir rate during preparation, microsphere size, drug loading and PLA M.W. on the kinetics of cephalexin release were investigated.

3.2.3 Determination of Encapsulation Efficiency

An amount of microspheres (10 mg) was added to 5 ml of methylene chloride, which dissolved the polymer but not the drug. Water (30 ml) was then mixed with the organic phase, dissolving the drug. The phases were separated and the process repeated twice. Over 90% of the drug was removed after the initial extraction, and the aqueous phase was analyzed spectrophotometrically at 260 nm (Beckman, Model 25 spectrophotometer). Experiments were run at least in triplicate. The encapsulation efficiency was determined as the ratio of the amount

analyzed to the initial amount of cephalexin added.

3.2.4 Release Studies of Cephalexin (CPX) from Microspheres

Microspheres (containing 6 mg equivalent weight of cephalexin) were dispersed in 500 ml of water or phosphate buffer at pH 7.4 (μ=0.17) at 37°C, and stirred at 100 rpm using a spin-filter dissolution test apparatus (nominal porosity, 1μm) (Clow-Coffman, Kansas City, Ks). The medium was continuously circulated through the spectrophotometer and absorbances were recorded at 260 nm. Concentrations of cephalexin released were determined as a function of time from a calibration curve. At the end of the experiment, the microspheres were filtered (Whatman No. 5, nominal porosity 2.5 μm) then dissolved in methylene chloride to determine the amount of drug in the microspheres remaining to be released. The cephalexin was extracted into water as described above and analyzed. The total amount of drug released into the medium and the amount determined in the microspheres at the end of each experiment accounted for 100 percent of the drug. Experiments were conducted in triplicate.

3.3 RESULTS AND DISCUSSION

The coacervation process of microencapsulation can produce microspheres or microcapsules having a wide range of properties (12-14). The characteristics of these formulations are dependent on several factors, including type of polymer, shape and the size of the suspended drug particles, the polymer:drug ratio, the type of nonsolvent and its rate of addition, and the rate of stirring during

preparation (12-14). Raw and spray-dried cephalexin monohydrate were each microencapsulated with PLA under identical conditions. However, the average particle size of the spray-dried cephalexin was considerably smaller than the raw cephalexin (Figure 3.1A and 3.1B). The appearance of the microspheres produced (Figure 3.2A and 3.2B) indicate that aggregates of drug and polymer coalesced to form a highly porous matrix giving a sponge-like appearance at the surface. The size distribution of the microspheres was larger than that of the naked crystals of cephalexin in both cases (not shown). PLA microspheres of other drugs have been reported to have similar morphologies (9-11). The kinetics of release of cephalexin from 33% w/w microspheres was less for raw than for spray-dried cephalexin (Figure 3.3) because of the larger particle size of raw cephalexin. However, spray-dried cephalexin was selected for further formulation studies because of the small, more uniform particle size of this material.

The microencapsulation process yielded an encapsulation efficiency of about 96 percent cephalexin as indicated in Table 3.1 and 3.2 for 33% w/w loading, with a batch-to-batch variation of 0.6 percent after repeated sampling and comparisons of sieve-size fractions, indicating a highly reproducible process. The amount of sample prepared in each of the sieve-size fractions was also very reproducible, yielding 42, 33, and 25% w/w of a sample as 40/60, 60/120, and under 120 mesh, corresponding to 250-425 μm, 125-250 μm, and under 125 μm, respectively (Table 3.3).

The release profiles of the three sieve-size fractions are compared in Figure

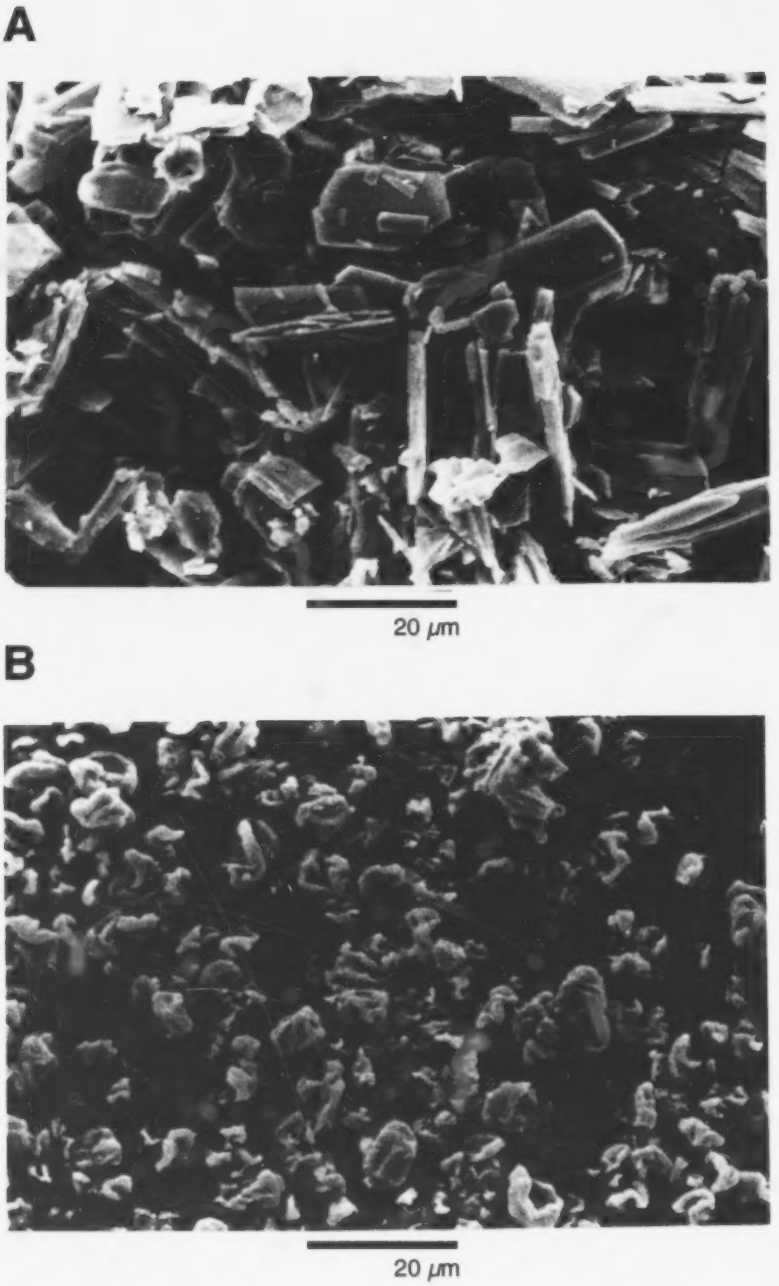

Figure 3.1 Scanning electron micrographs of cephalexin particles: **A**. Raw cephalexin; **B**. Spray-dried cephalexin, x 1000 magnification.

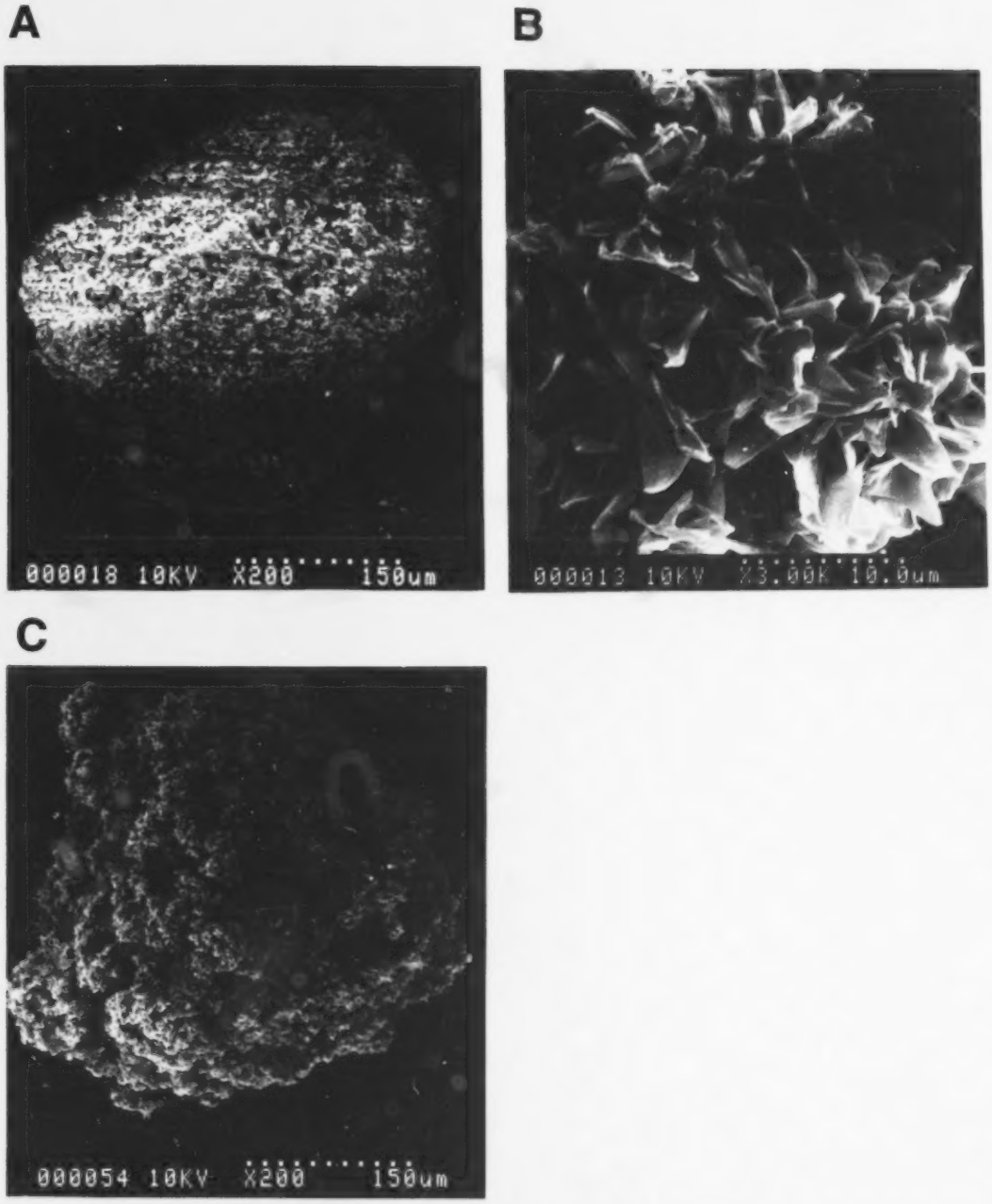

Figure 3.2 Scanning electron micrographs of 33% w/w cephalexin microspheres: **A**. 40/60 mesh size prepared at 400 rpm, x 200 magnification; **B**. same as A at x 3000 magnification; **C**. 40/60 mesh size prepared at 100 rpm, x 200 magnification.

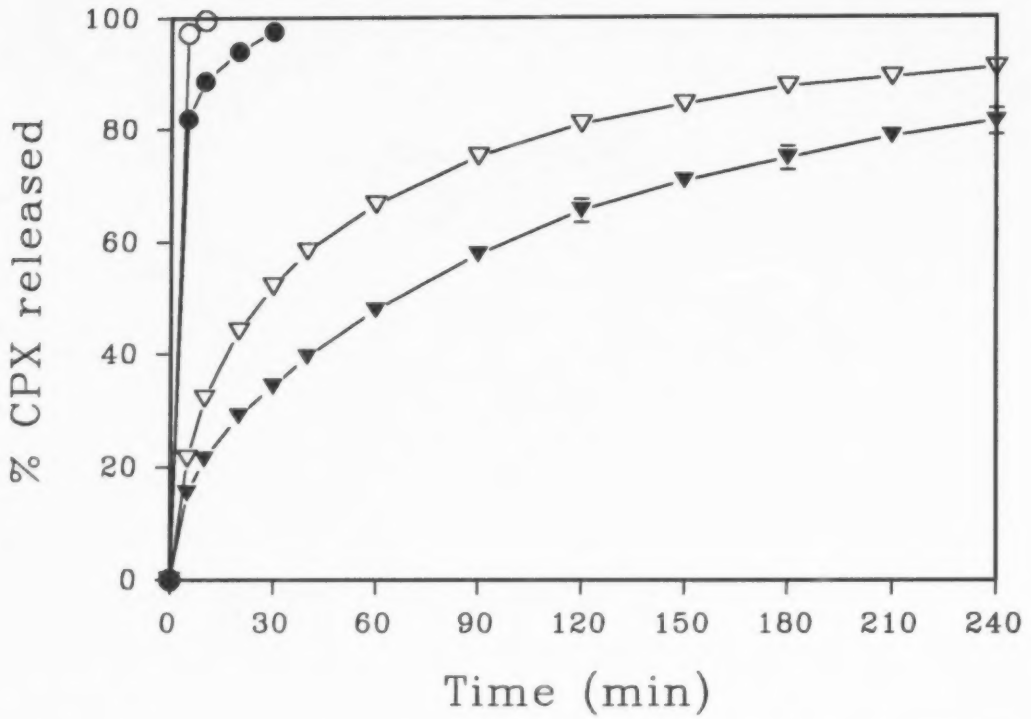

Figure 3.3 Comparison of the dissolution behavior of raw (closed symbols) and spray-dried (open symbols) CPX. (O), free CPX; (∇), 33% w/w PLA microspheres.

Table 3.1 Batch-to-batch variation of encapsulation efficiency of CPX in PLA microspheres[1]

Batch Number	Sample Number				Mean (±SEM)
	1	2	3	4	
1	96.9	97.5	95.9	96.4	96.7(0.4)
2	94.8	95.3	95.4	95.2	95.2(0.2)
3	95.9	96.7	94.9	95.4	95.7(0.4)
4	96.6	95.0	96.2	97.1	96.2(0.5)
5	96.3	94.8	95.1	96.1	95.6(0.4)
6	94.1	95.2	94.6	95.9	95.0(0.4)
Average					95.7(0.3)

SEM, standard error of the mean.

[1] 33% w/w spray-dried CPX; 40/60 mesh size.

Table 3.2 Variation of the encapsulation efficiency of CPX in PLA microspheres for different sieve size fractions[1].

Batch Number	Sieve Number (passed/retained) 120/0	60/120	40/60	Mean (±SEM)
1	94.8	96.3	95.1	95.4(0.5)
2	96.1	95.0	96.6	95.9(0.6)
3	94.6	96.1	95.4	95.4(0.2)
4.	97.5	95.9	96.8	96.8(0.3)
Average				95.9(0.4)

[1] 33% w/w spray-dried CPX.

Table 3.3 Variation in the percent of a batch of CPX microspheres separated as three sieve size fractions[1].

Batch	Sieve Size Range (passed/retained)		
Number	Under 120	60/120	40/60
1	25.9	32.6	41.5
2	24.1	34.8	41.1
3	24.6	33.1	42.3
4	23.2	33.9	42.9
5	24.8	33.4	41.8
6	25.1	32.8	42.1
Mean size distribution(SEM)	24.6(0.4)	33.4(0.3)	42.0(0.2)

[1]33% w/w spray-dried CPX.

3.4, exhibiting apparent second order kinetics (Figure 3.5) according to Eq. 1:

$$\frac{W}{W_e(W_e - W)} = k_2 t \qquad (1)$$

where W is the amount of drug in solution at time, t, and W_e is the amount of drug in solution at equilibrium, which is considered to be the total amount of drug in the microspheres since the dissolution profiles indicate 100 percent of cephalexin is dissolved after a sufficient period of time. Also, the initial dissolution rate (IDR) = $k_2W_e^2$ is inferred in Eq. 1 under the conditions that at t = 0, W = 0. The correlation coefficient (r^2) of all second order plots was > 0.995.

The apparent second order release rate constant, k_2, for microspheres prepared at stirring rates ranging from 100 to 500 rpm are listed in Table 3.4. These data permitted the selection of the optimum stirring rate, 400 rpm, which yielded the smallest k_2, the longest T_{50}, and which provided the most uniform microsphere characteristics. The microsphere characteristics shown in Figure 3.1E were produced at a stirring rate of 100 rpm, clearly indicating a shape and morphology of a less compact microsphere. The T_{50} of this microsphere was one-seventh and one-half of that obtained for 250-425 μm and 125-250 μm sieve-size fractions of microspheres prepared at 400 rpm, respectively. The second order release kinetics suggests a dissolution-controlled process of drug leaching from the pores of the microspheres, being a function of the amount of cephalexin remaining to be dissolved and the effective surface area of the drug particles (8).

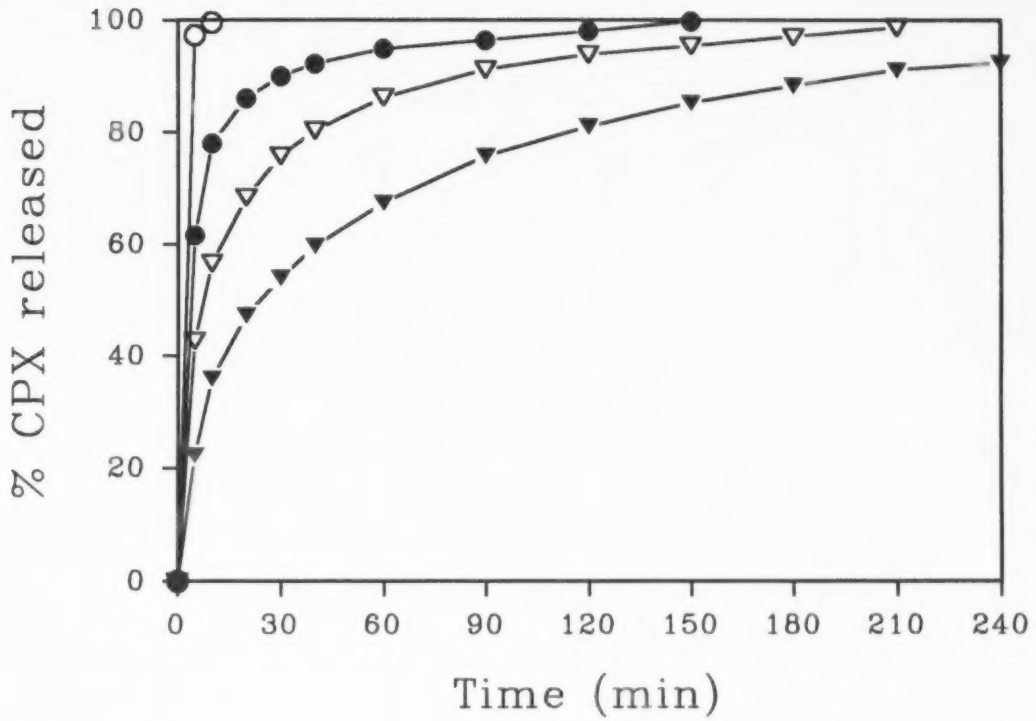

Figure 3.4 Release of CPX from 33% w/w PLA microspheres as a function of microsphere size at 37°C, pH 7.4. (○), free CPX; (●), <125 μm; (▽), 125-250 μm; (▾), 250-425 μm. The dissolution of an equivalent weight of spray-dried CPX powder is shown for comparison.

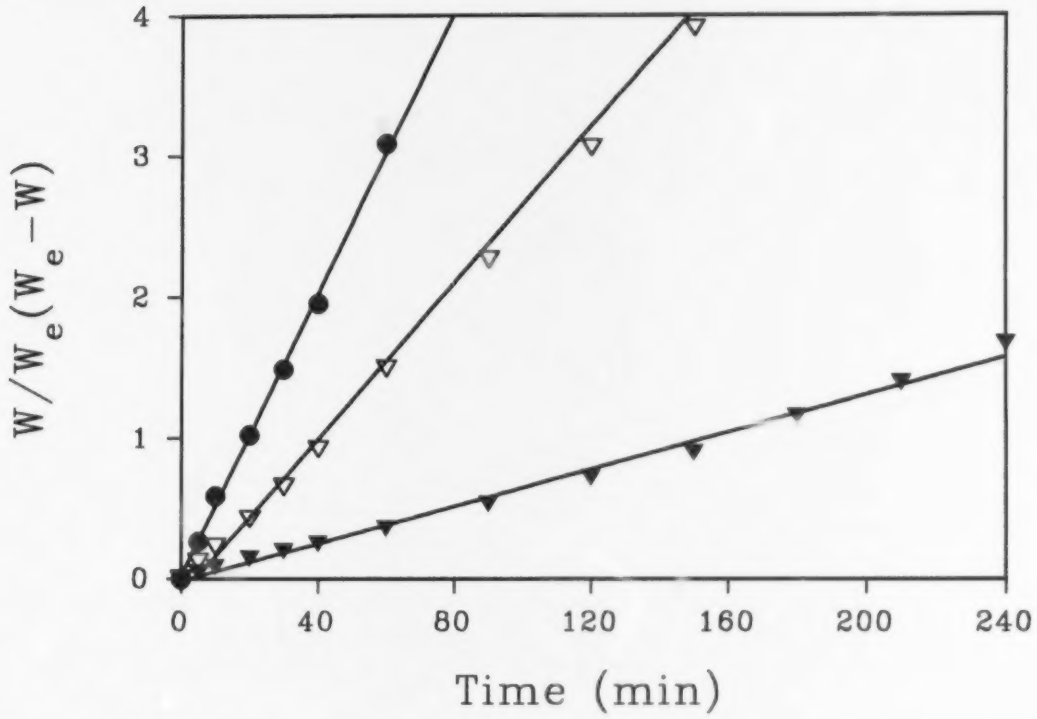

Figure 3.5 Second order plots of the release of CPX from 33% w/w PLA microspheres. (●), <125 μm; (▽), 125-250 μm; (▾), 250-425 μm.

As the drug loading of the microspheres increased from 23% to 33% w/w, the rate of release of cephalexin decreased, but the release rate increased when the loading increased from 33 to 43% w/w (Figure 3.6 and 3.7). This behavior would appear to be related to the conditions of microsphere preparation in which a fixed concentration of polymer was used. At low cephalexin concentration under constant stirring conditions, the efficiency of coacervate deposition on the small surface area exposed by the drug particles is low and particles of polymer are also formed. The efficiency increases, however, as the total surface area increases to an optimum value, apparently at 33% w/w. Thereafter, the total surface area produced by the cephalexin particles becomes incompletely covered by the limited amount of polymer within the matrix of the microspheres and, consequently, a greater dissolution rate is observed. Deposition of polymer coacervate on the particle surfaces and agglomeration of these within the microspheres is obviously affected by the system dynamics during preparation as indicated by the results in Table 3.4. At 400 rpm, the microspheres which formed yielded the slowest release whereas stirring at slower or faster rates caused microsphere formation to be less compact.

Reducing PLA molecular weight from 100,000 to 50,000 produced a faster release rate (Figure 3.8). However, using PLA/Poly(glycolic acid) (85:15) copolymer of 100,00 M.W. to prepare microspheres did not significantly alter the release profile from that obtained with PLA 100,000 M.W.

The IDR and k_2 values shown in Table 3.5 indicate the quantitative effect of

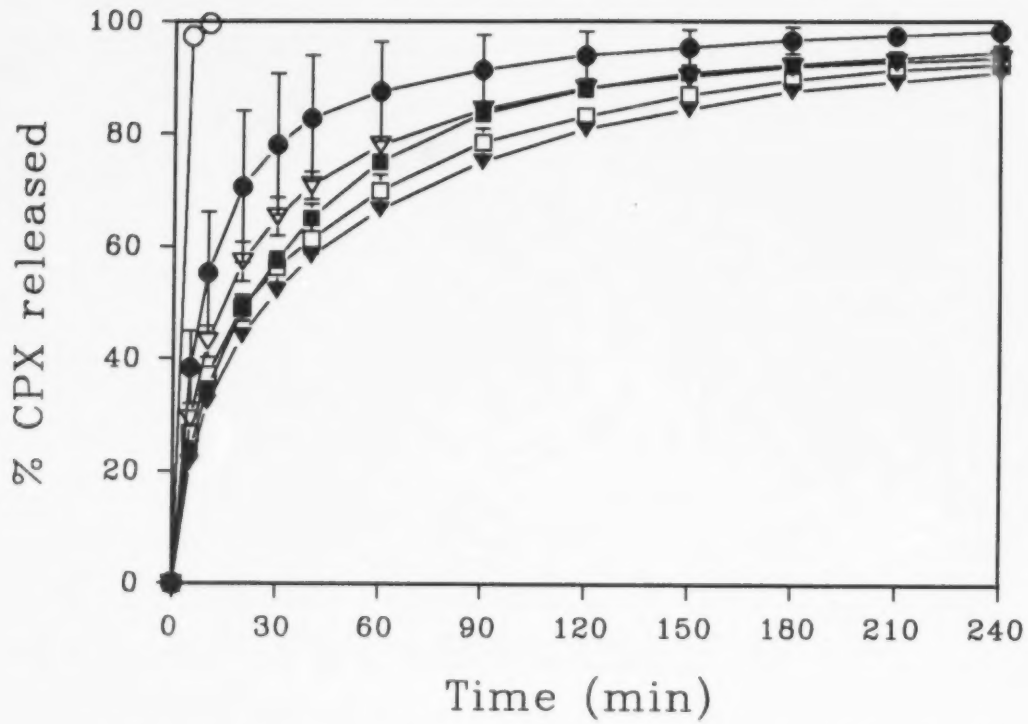

Figure 3.6 Release of CPX from PLA microspheres as a function of drug loading (% w/w). (○), free CPX; (●), 43; (▽), 37; (▾), 33; (□), 29; (■), 23. The dissolution of an equivalent weight of spray-dried CPX powder is shown for comparison.

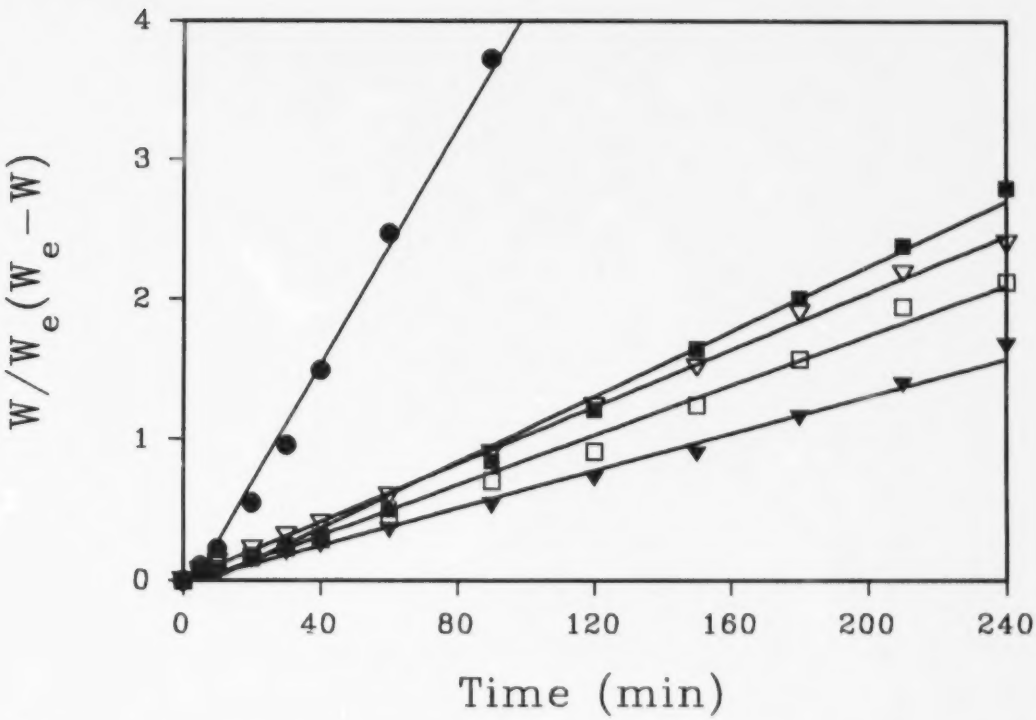

Figure 3.7 Apparent second order release kinetics of CPX from PLA microspheres as a function of drug loading (% w/w). (●), 43; (▽), 37; (▾), 33; (□), 29; (■), 23.

Table 3.4 Apparent second order release kinetics of CPX from 33% w/w PLA microspheres at 37°C, pH 7.4 as a function of stirring rate during preparation

Rate of Stirring (rpm)	k_2[1]	T_{50}[2]	
	250-425 μm	250-425 μm	125-250 μm
100	17.3	4.00	3.50
200	11.5	9.50	4.00
300	10.0	15.0	5.00
400	6.21	28.0	7.00
500	16.6	16.0	5.50

[1]Apparent second order release rate constant x 10^3, $mg^{-1}min^{-1}$.

[2]Time for 50 percent of CPX to be released, min.

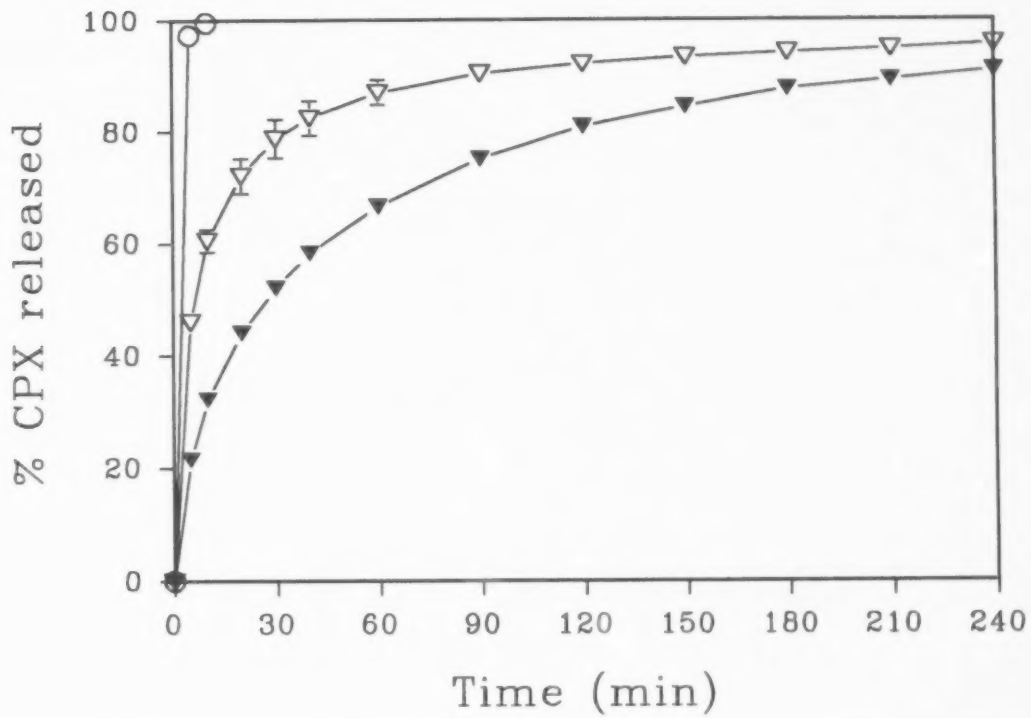

Figure 3.8 Comparison of the effect of PLA M.W. on the release of CPX from 33% w/w microspheres. (O), free CPX; (▾), 100,000 M.W.; (∇), 50,000 M.W. The dissolution of an equivalent weight of spray-dried CPX powder is shown for comparison.

Table 3.5 Kinetics of release of CPX from PLA microspheres at 37°C., pH 7.4 as a function of loading

CPX (% w/w)	k_2[1]	T_{50}[2]	IDR[3]
23	11.7	22	0.42
29	8.89	23	0.32
33	6.21	28	0.22
37	10.2	19	0.37
43	42.2	8.0	1.52

[1]Apparent second order rate constant, x 10^3, $mg^{-1}min^{-1}$.

[2]Time for 50% of CPX to be released, min.

[3]Initial dissolution rate, mg min^{-1}

loading on the kinetics of cephalexin release. Thus, approximately the same kinetics of release were obtainable from 33% w/w, 125-250 μm. 100,000 M.W. PLA; 33% w/w, 250-425 μm, 50,000 M.W. PLA; and 43% w/w, 250-425 μm, 100,000 M.W. PLA microspheres. With additional data it is likely that other similar comparisons could be made. In each of these formulations cephalexin concentrations increased for at least 4 h compared to only 10 min for complete dissolution of an equivalent weight of cephalexin powder. It is concluded that the formulation of microspheres of cephalexin with PLA yields sustained release kinetics that can be varied to address the requirements of treating localized infections.

3.4 REFERENCES

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CHAPTER 4

FORMULATION AND RELEASE KINETICS OF CIPROFLOXACIN HYDROCHLORIDE FROM BIODEGRADABLE POLYMERIC MICROSPHERES

A version of this chapter has been published in the Proceedings of the 20th International Symposium on Controlled Release of Bioactive Materials.

4.1 INTRODUCTION

There are numerous examples of formulation of antibiotics as sustained release preparations (1-7). Only a few reports, however, have described sustained release microparticles having intermediate release time scales for localized drug delivery of, for example, several hours (6) instead of several days or weeks (7). Over the shorter period, usually higher concentrations of drug are obtainable at the site of administration than from slow release dosage forms. This is an advantage using antibiotics at localized sites of infection since sub-potent levels of antibiotic can lead to the development of bacterial resistance. This problem is particularly acute when treating device-associated biofilm infections (8-11). Thus, a desirable goal is to obtain a sufficiently high antibiotic concentration and then maintain it until complete eradication has occurred.

It would be advantageous to prepare antibiotic microparticles with a biodegradable polymer because it is almost impossible to remove these completely from an injection or implantation site without surgery. Poly(lactic acid) has been approved for medical use as sutures, and many other applications and can be conveniently formulated as microparticulate drug delivery systems by well-established processes (12-15). Hence, this study was aimed at formulating ciprofloxacin, a fluoroquinolone antibiotic with broad spectrum activity (16), as polymeric microspheres intended for I.P. administration in the treatment of localized device-associated bacterial biofilm infections in patients undergoing continuous ambulatory peritoneal dialysis (CAPD).

4.2 EXPERIMENTAL

4.2.1 Materials

Poly (L-lactic acid) (PLA), (50,000, 100,000 and 300,000 M.W.) were obtained from Polysciences Inc. (Warrington, PA) and ciprofloxacin hydrochloride (CFX) was obtained from Sigma Chemical Co., St. Louis, MO. Methylene chloride, diethyl ether and all other chemicals were reagent grade.

4.2.2 Preparation of Microspheres

Microspheres were prepared using a coacervation procedure. Typically, CFX (100, 200, 300, 400, or 600 mg, < 250 μm) particle size were dispersed in 4 ml of methylene chloride by probe sonication (Heat Systems, Model W-375, Plainview, N.Y.) then transferred to a round bottom flask containing 6 ml of a solution of PLA in methylene chloride to make a final concentration of 40 mg/ml of PLA. Magnetic stirring was carried out at 400 rpm (Ikamag stir plate). Diethyl ether (36 ml) was added dropwise at a constant rate of 0.2 ml/min using a syringe pump (Sage Instruments, Boston, MA) for 3 h. The microspheres which formed were harvested by filtration, (Whatman No. 5, nominal porosity 2.5 μm), washed lightly with water then vacuum-dried over $CaCl_2$ in a desiccator. In those cases when a slightly-packed cake had formed on drying (microspheres prepared with 50,000 or 100,000 M.W. PLA) the sample was dispersed in diethyl ether, then the microspheres were filtered and dried. All products were sieve-sized into various fractions (U.S. standard sieves).

4.2.3 Determination of Encapsulation Efficiency

A 10 mg sample of microspheres was added to 5 ml of methylene chloride in which the polymer dissolved but not the drug. The organic phase was mixed with 30 ml phosphate buffer solution (PBS) pH 7.4, μ=0.17 which after a short period of mixing, the CFX was extracted and dissolved. The phases were separated and the process repeated twice. CFX was analyzed spectrophotometrically in the aqueous phase at 328 nm (Beckman, Model 25) and concentrations of CFX were obtained from a calibration curve. Experiments were conducted at least in triplicate and the results averaged. The encapsulation efficiency was calculated as the fraction of amount of drug in the microspheres determined from analysis to amount of drug added initially.

4.2.4 Release Studies of Ciprofloxacin (CFX) from Microspheres

Microspheres (containing 7 mg equivalent weight of CFX) were dispersed in 500 ml of PBS at 37°C and stirred at 100 rpm using a spin-filter dissolution test apparatus (nominal porosity, 1 μm) (Clow-Coffman, Kansas City, Ks). The medium was continuously circulated through the spectrophotometer and absorbances were recorded up to 7 h. Concentrations of CFX released were determined from a calibration curve as a function of time. At the end of the experiment, the microspheres were filtered (Whatman No. 5, nominal porosity 2.5 μm) and then dissolved in methylene chloride. The CFX was extracted into PBS as described above and analyzed. The total amount of drug released into the medium and the amount determined in the microspheres at the end of each experiment accounted

for 100 percent of the drug. Experiments were conducted in triplicate.

4.3 RESULTS AND DISCUSSION

CFX is practically insoluble in diethyl ether but has a solubility of 20 μg/ml in methylene chloride. Actual drug loadings were close to the theoretical drug loadings with encapsulation efficiencies > 90%. Some drug was lost during the washing of the microspheres with water during harvesting, the drug having a water solubility of about 35 mg/ml.

When a batch of microspheres is prepared in this manner, there is the possibility of batch-to-batch variation in the particle size distribution. The results in Table 4.1 confirm that, at least for microspheres prepared with 50,000 and 100,000 M.W. PLA, there is no significant difference in the distribution between batches, approximately 38 percent of a batch existing in the 40/60 and <120 sieve size fractions, respectively. On the other hand, a batch of microspheres prepared with 300,000 M.W. PLA occurred predominately as the 60/120 sieve size fraction at the expense of the 40/60 fraction and the reproducibility was slightly less.

Scanning electron microscopic (SEM) examination (Figure 4.1) revealed near spherical shapes of the microspheres consisting of 50,000 or 100,000 M.W. PLA but irregular shapes for those consisting of 300,000 M.W. PLA. There was a corresponding lower tendency of the latter microspheres to aggregate during preparation. A higher rate of coacervation of the 300,000 M.W. PLA may also have contributed to the appearance and characteristics of the microspheres.

Table 4.1 Percent of 33% w/w CFX microsphere batch weight separated into various sieve size fractions for microspheres of three PLA molecular weights.

M.W. of PLA	Sieve Number (passed/retained)		
	Under 120 (<125 μm)	60/120 (125-250 μm)	40/60 (250-425 μm)
50,000	35.7(0.5)	22.9(0.5)	41.8(0.3)
100,000	39.0(0.5)	24.0(0.6)	37.1(0.3)
300,000	34.0(0.8)	46.0(1.0)	19.9(1.1)

Standard error of the mean (SEM) shown in brackets.

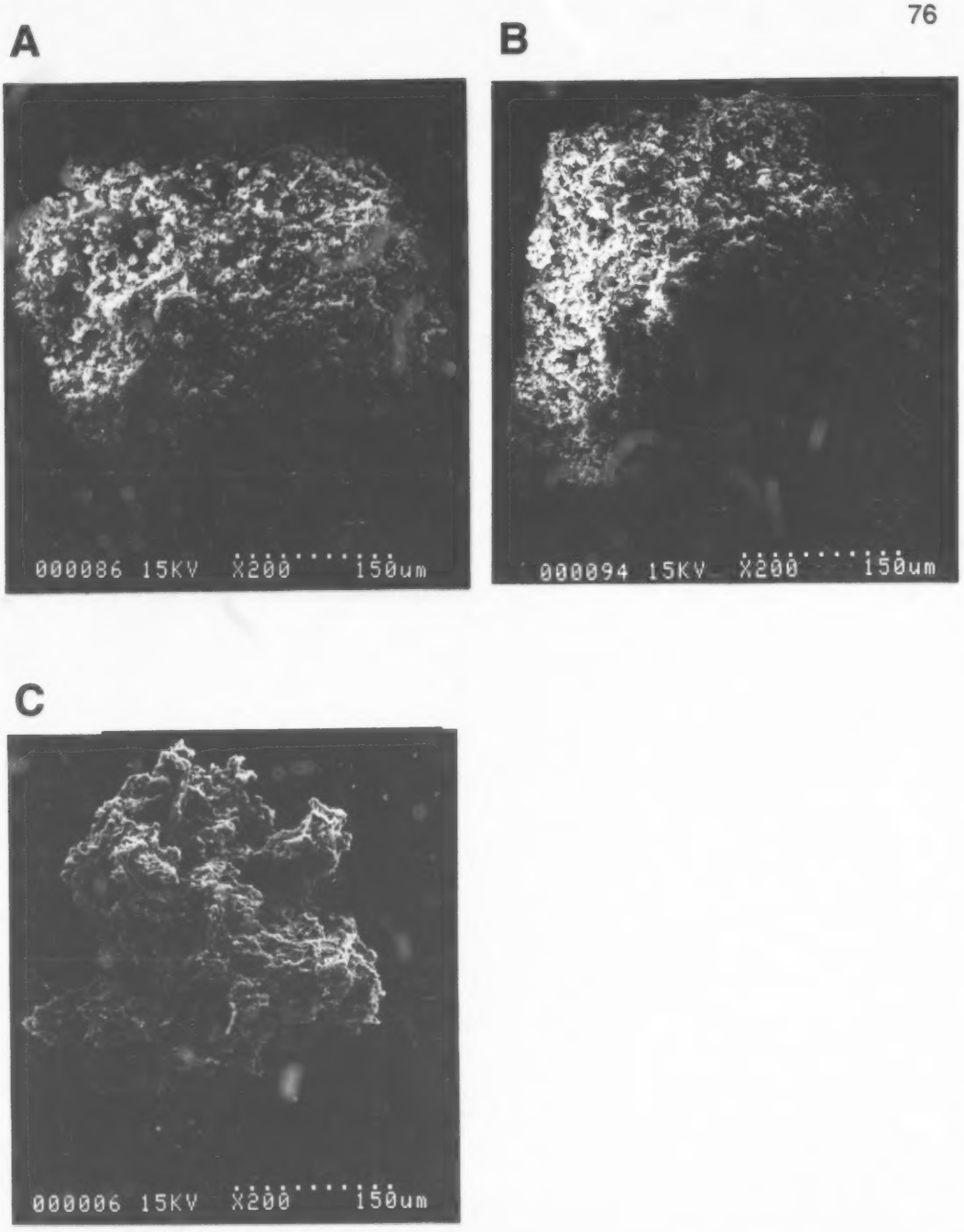


Figure 4.1 Scanning electron micrographs of 33% w/w ciproflaxacin microspheres prepared with, A. 50,000 M.W.; B. 100,000 M.W.; and C. 300,000 M.W. PLA (x 200 magnification

The release of CFX from 250-425 μm microspheres of 100,000 M.W. PLA as a function of drug loading is depicted in Figure 4.2. There was a faster initial rate of release of CFX at low loadings, which decreased with increased loadings up to 60% w/w, a behavior that is opposite to that often seen with other microparticulate systems (7,17). Similar release profiles were observed by Bodmeier et al. (18) for release of quinidine through poly(DL-lactide) films of various polymer composition. Furthermore, the influence of drug loading is relatively minor with these microspheres. It is apparent from the time scale of release that the kinetics are not dominated by diffusion of drug through a polymer phase, which might be expected from their appearances (Figure 4.1). Hence, the data were fitted to various dissolution kinetic models for multiparticulate systems including first order (19), second order (20), cube root law (21), and Weibull distribution theory (22). Only the second order model provided a satisfactory fit of the data according to Eq. 1:

$$\frac{W}{(W_e - W)\,W_e} = k_2 t \qquad [1]$$

where, W is the weight of drug in solution at time, t, W_e is the weight of drug in solution at equlibrium, which is assumed to be equal to the weight of CFX in the microspheres at, t = 0, since 100% of the drug is dissolved after a sufficient period of time, and k_2 is the apparent second order dissolution rate constant.

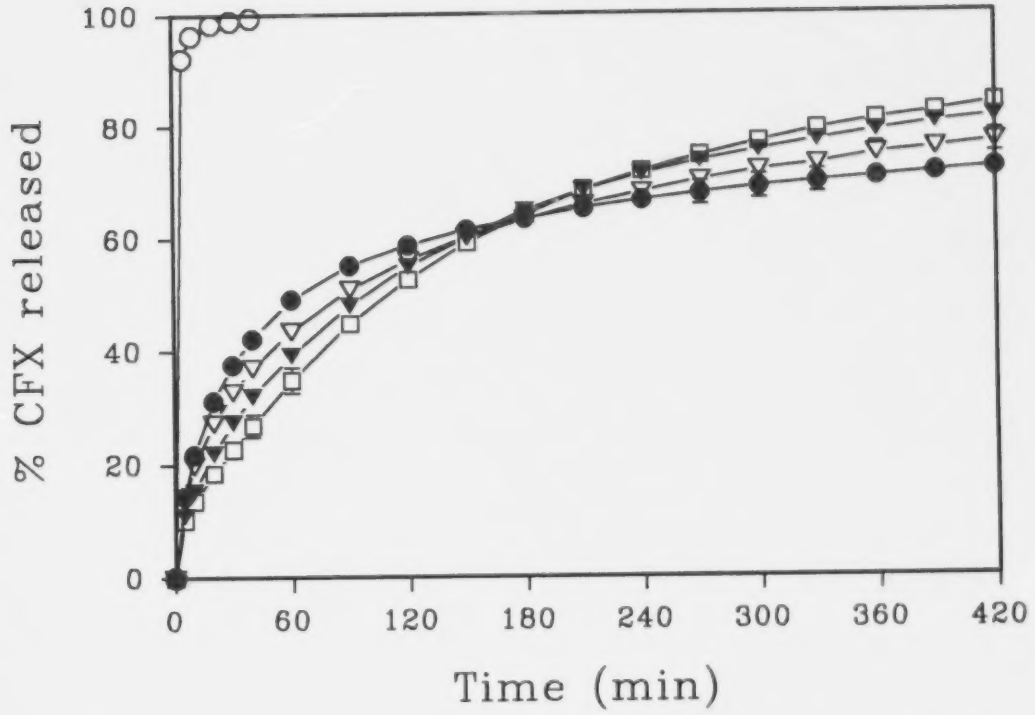


Figure 4.2 Release of CFX from PLA microspheres (250-425 μm) as a function of drug loading (% w/w) at 37°C, pH 7.4. (●), 20; (▽), 33; (▾), 50; (□), 60. The dissolution of an equivalent weight of CFX powder (○) is shown for comparison.

The first derivation of Eq. 1 is :

$$\frac{dW}{dt} = k_2 (W_e - W)^2 \qquad [2]$$

The dissolution rate from a solid by the Noyes-Whitney equation is given as:

$$\frac{dW}{dt} = k . S [(X - W) - (X - W_e)] \qquad [3]$$

where S is the surface area of the dissolving solid assumed constant and X is the initial weight of the solid present. It is reasonable to assume that the surface area available at any time is approximately proportional to the amount of solid drug remaining to be dissolved. Thus the effective surface area may be written in terms of W as:

$$S = k_s [(X - W) - (X - W_e)] \qquad [4]$$

where k_s is the proportionality constant. When $W = W_e$, then $S = 0$. Thus effective surface should be zero at equilibrium concentration and no more solid dissolves. Substituting the right-hand side expression of Eq. 4 into Eq. 3:

$$\frac{dW}{dt} = k . k_s (W_e - W)^2 \qquad [5]$$

$$\frac{dW}{dt} = k_2 (W_e - W)^2 \qquad [2]$$

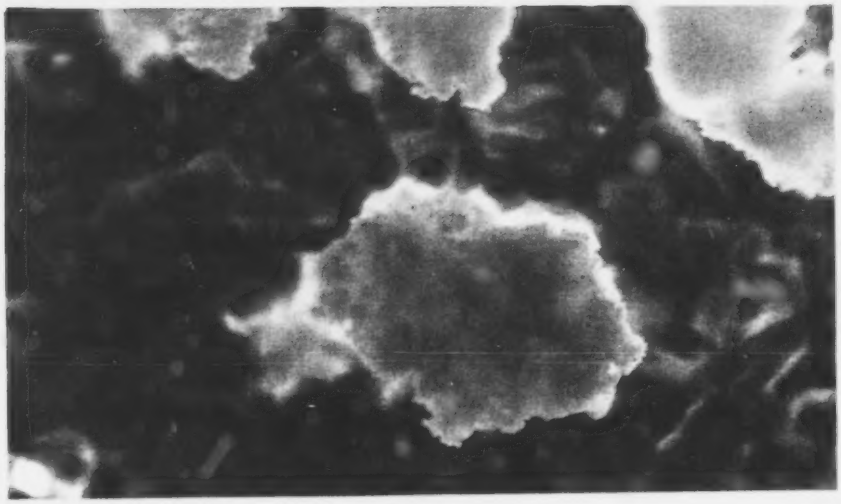
Thus an apparent second order rate of dissolution should be observed with

changing surface area. The production of microspheres resulted in drug particles which were coated with polymer to various extents. These coalesced together to form a matrix consisting of drug and polymer. This has been confirmed by electron micrographs of the internal structure of the microsphere (Figure 4.3). By obeying second order kinetics, the release of CFX from these microspheres was not only dependent upon the surface area of exposed drug, but also on the weight of drug available for dissolution (20). Typical second order plots are shown in Figures 4.4, 4.5, and 4.6. It can be seen that a decrease in particle size, an increase in drug loading or a decrease in PLA M.W. resulted in an increase in release rate. At 20% w/w drug loading, the initial release up to 1 h does not appear to follow second order kinetics. The initial mechanism of release is not known but appears to be due to rapid release of the drug close to the surface of the microspheres (burst effect). After 1 h, the kinetics of release follow second order (Figure 4.4).

The effect of PLA concentration used to prepare the microspheres on release profiles was investigated. It was observed that an increase in PLA concentration from 2 to 4% resulted in a decrease in release rate but the trend reversed with further increase in PLA concentration (8%) (Figure 4.7). Jalil and Nixon (23) observed an optimum slowing down of release with polylactic acid concentration of 3.33%. It would appear that for polymer coating to be effectively deposited on the constant surface area exposed by the drug particles, an optimum polymer concentration would be needed.

A comparison between the release profiles of three sieve size fractions are

A



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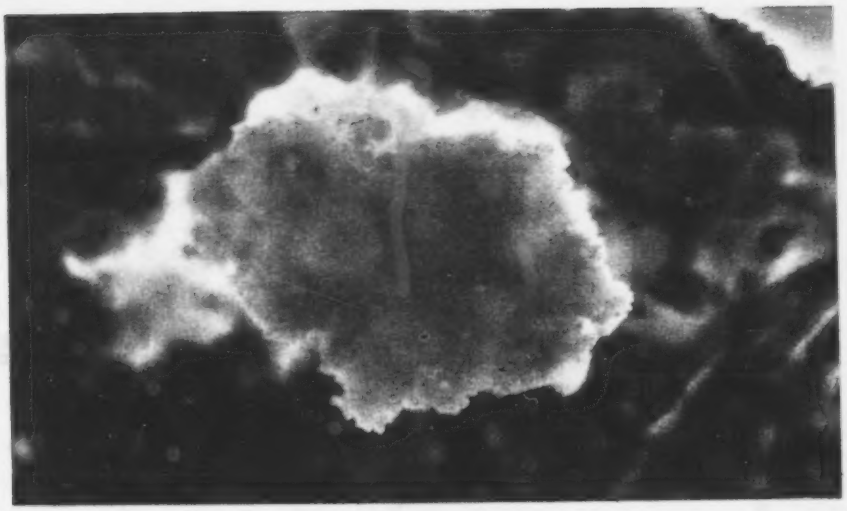

Figure 4.3 Light micrographs of cross-sections of PLA microspheres of ciprofloxacin. **A**. (x 130 magnification); **B**. (x 170 magnification).

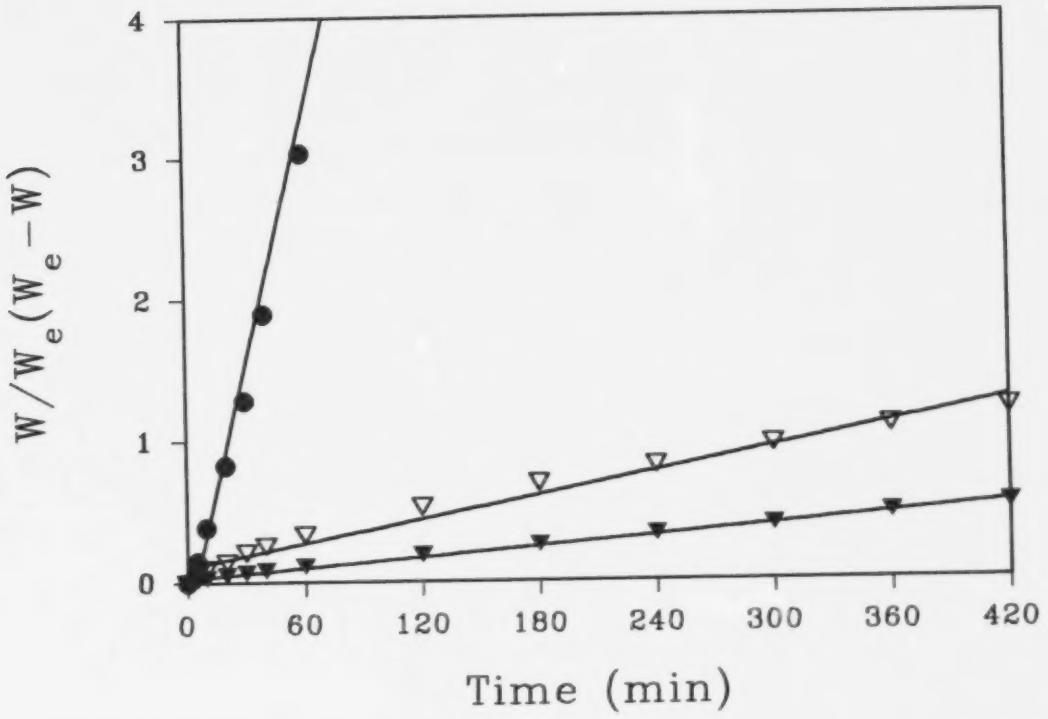

Figure 4.4 Second order plots of the release of CFX from 33% w/w PLA (100,000 M.W.) microspheres as a function of particle size. (●), <125 μm; (▽), 125-250 μm; (▾), 250-425 μm.

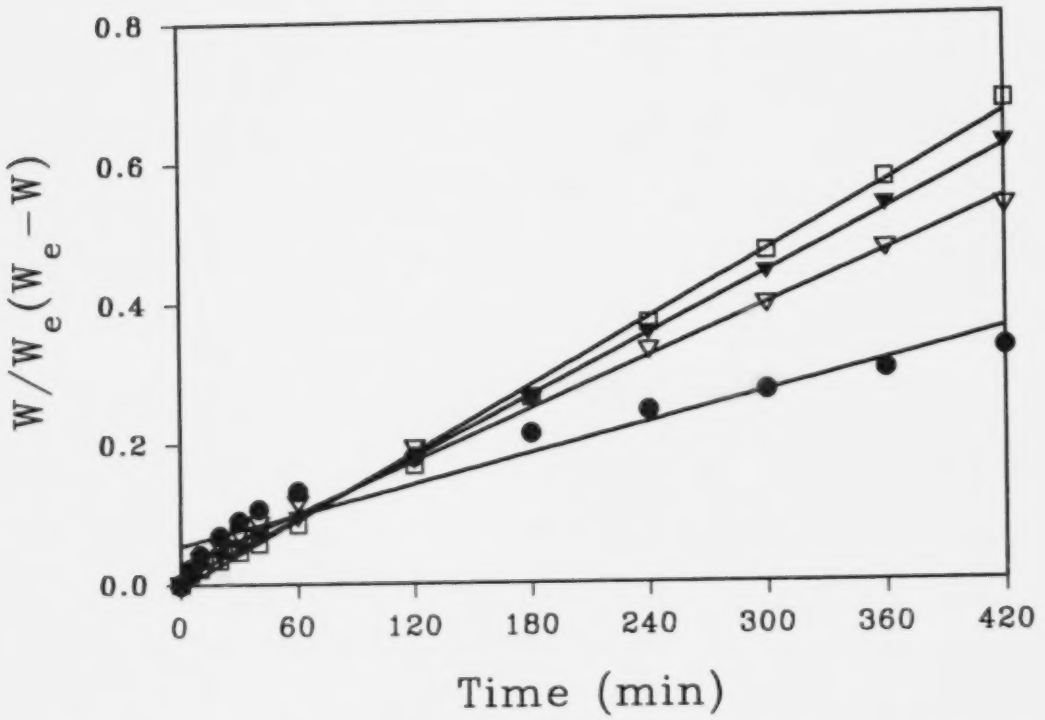

Figure 4.5 Second order plots of the release of CFX from PLA microspheres as a function of drug loading (% w/w). (●), 20; (▽), 33; (▾), 50; (□), 60.

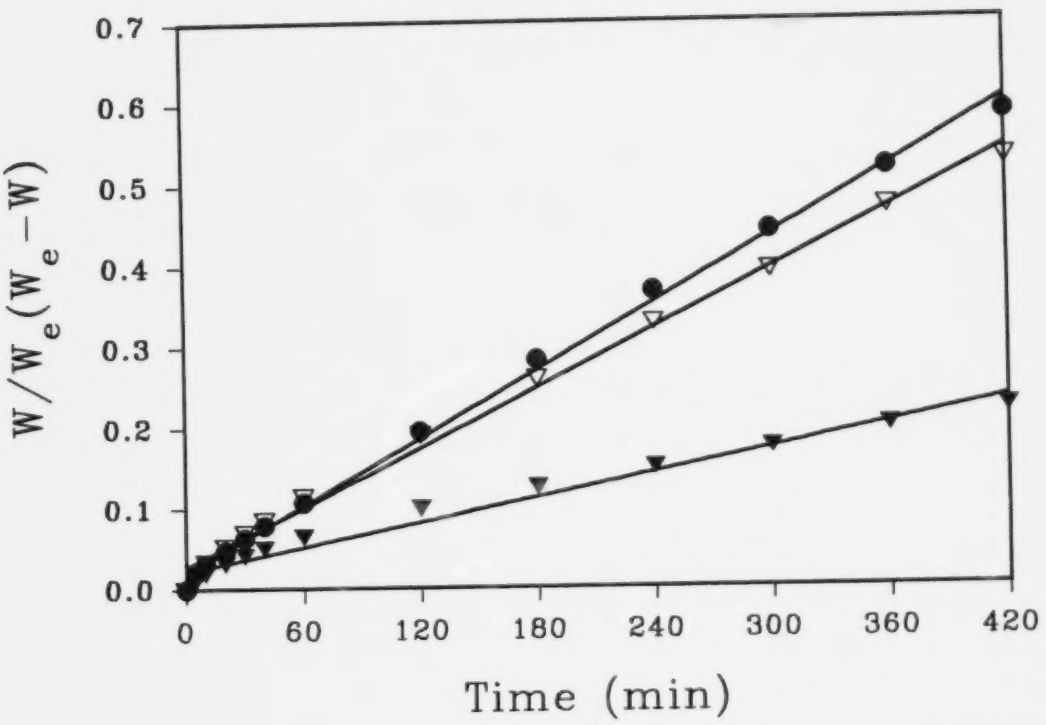

Figure 4.6 Second order plots of the release of CFX from PLA microspheres of size range 250-425 μm, as a function of the M.W. of PLA. (●), 50,000; (▽), 100,000; (▾), 300,000.

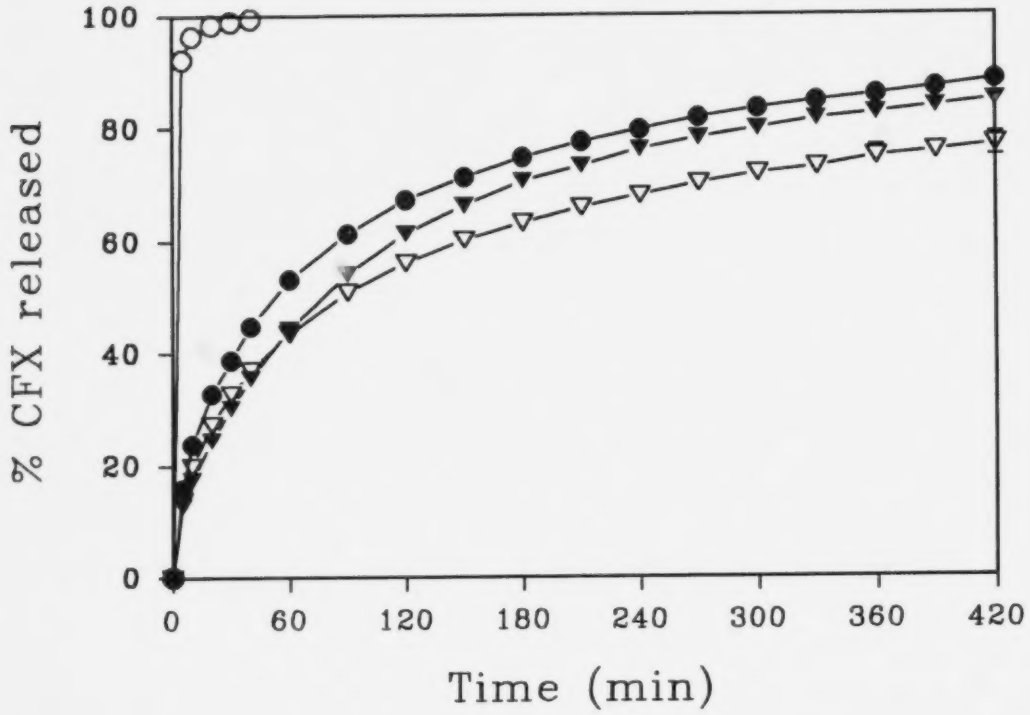

Figure 4.7 Release CFX from PLA (100,000 M.W.) microspheres (250-425 μm) as a function of the concentration (% w/v) of PLA used to prepare the microspheres. (●), 2; (▽), 4; (▾), 8.

shown in Figures 4.8 and 4.9 for microspheres prepared with PLA 100,000 and 300,000 M.W., respectively. The effect of particle size appears to more significant with microspheres prepared with PLA 50,000 or 100,000 M.W. An increase in the effective surface area due to decreased microsphere size resulted in an increase in release rate. Table 4.2 compares the kinetics of release of CFX from PLA microspheres for three molecular weight grades of PLA of similar particle size range. The apparent second order release rate constant, k_2, decreased 3-fold whereas the observed T_{50} increased 4-fold by increasing the PLA M.W. from 50,000 to 300,000. A plot of k_2 versus M.W. of PLA is linear (Figure 4.10) and the calculated T_{50} is slightly higher than the observed T_{50} (Table 4.2). Although the mechanism of molecular weight control of the release is unclear, it is definitely related to the coating efficiency of the drug particles. The microspheres delivered CFX for a longer period of time compared to dissolution of the free drug powder.

The aim of this study was to develop sustained release of ciprofloxacin for intraperitoneal administration through the peritoneal catheter for the treatment of biofilm infections in patients undergoing CAPD. The residual peritoneal dialysate which needs to be removed, every 8 hours, limits the time required for the prolongation of release. The adminstration of CFX in solution at the clinic is not effective in treating biofilm related infections in patients undergoing CAPD (24,25) due to the short absorption half-life of most antibiotics including CFX in the peritoneum. This would require the administration of a sustained release product where a substantial amount of drug can be released during this period to offset

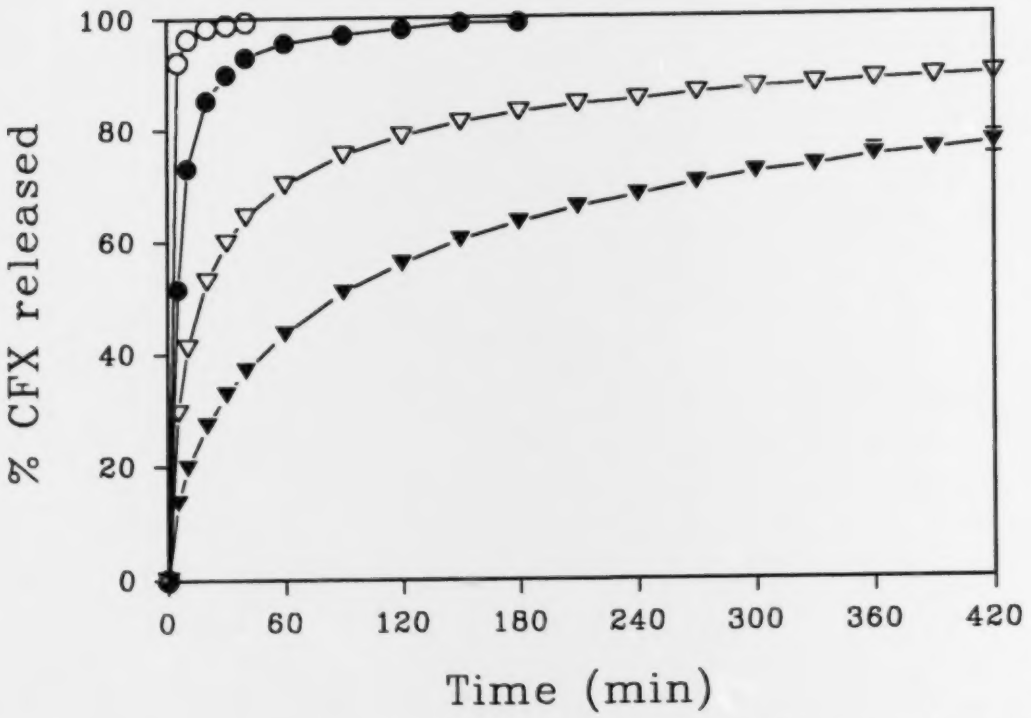

Figure 4.8 Release of CFX from 33% w/w PLA (100,000 M.W.) microspheres as a function of microsphere size. (●), <125 μm; (▽), 125-250 μm; (▾), 250-425 μm.

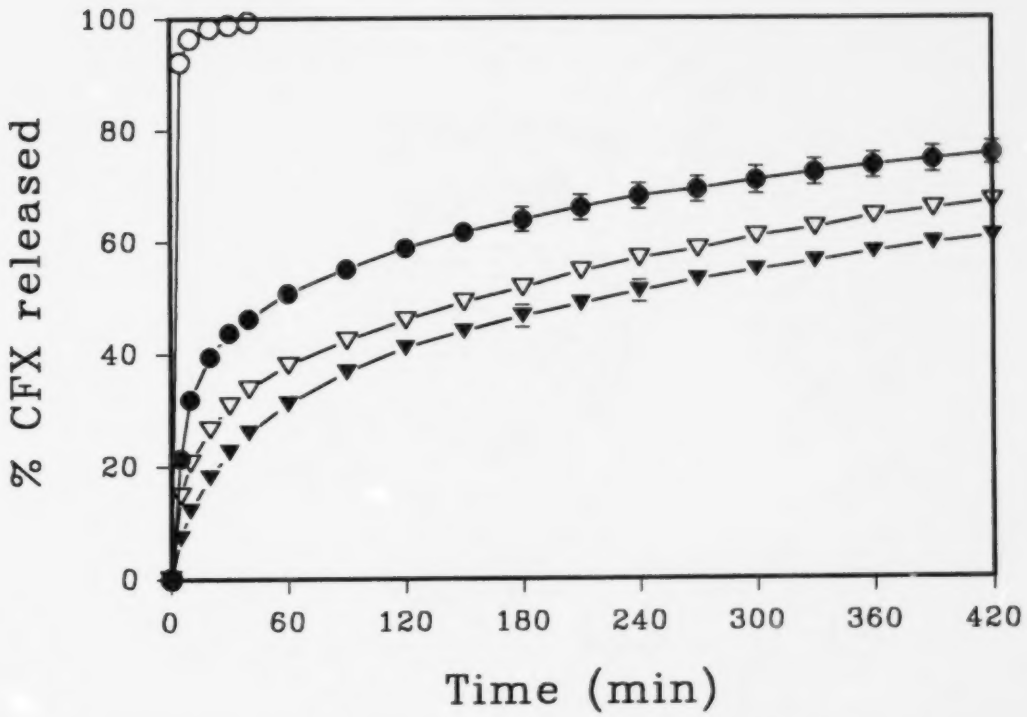

Figure 4.9 Release of CFX from 33% w/w PLA (300,000 M.W.) microspheres as a function of microsphere size. (●), <125 μm; (▽), 125-250 μm; (▾), 250-425 μm.

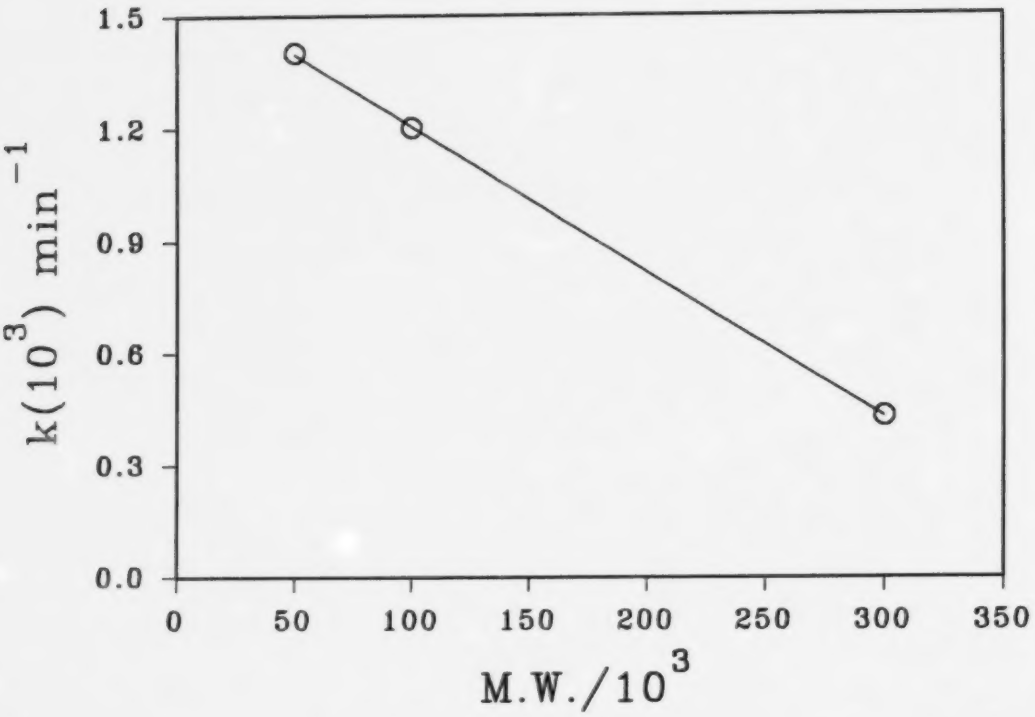

Figure 4.10 Linear dependence of the second order release rate, k_2, for release of CFX from PLA microspheres as a function of M.W of PLA.

Table 4.2 Kinetics of CFX release from PLA microspheres (250-425 μm) containing 33% w/w of CFX as a function of M.W..

M.W. of PLA	k_2[1]	Calculated T_{50}[2]	Observed T_{50}
50,000	1.39	103	72(4)
100,000	1.23	116	91(4)
300,000	0.43	333	270(6)

[1]apparent second order release rate constant, x 10^3, ($mg^{-1}min^{-1}$).

[2]time for 50% of CFX to be released. Standard error of the mean (SEM) shown in brackets.

loss from the peritoneum. The present studies involving CFX have yielded evidence that microspheres can be prepared which can be formulated having a variety of release rates of the drug by selecting the desired sieve-size range, drug loading, and M.W. of PLA which could release the drug at high concentrations. This will be desirable to fight bacterial biofilm infections which require about 10 times the dose needed to kill planktonic cells of the same organism (26,27)

The PLA can be removed with the residual peritoneal dialysate at the end of the dwell time. Moreover, the polymer degrades to non toxic histocompatible products and therefore empty microspheres which are not recovered with the residual dialysate should not pose any health hazard. Microspheres such as described in this paper, can release about 80% of the total drug in 7 h. In addition, the yield of microsphere production and efficiency of encapsulation are high, >90%. It is possible that microspheres of CFX can be selected to deliver antibiotic continuously during the dwell time of the peritoneal dialysate which would be effective to eradicate aged bacterial biofllms responsible for recurrent peritonitis.

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CHAPTER 5

FORMULATION TESTING OF SUSTAINED RELEASE MICROSPHERES IN AN OPEN CHEMOSTAT SYSTEM

A version of this chapter has been submitted for publication to Int. J. Pharm.

5.1 INTRODUCTION

Various methodologies have been employed to determine the kinetics of release of therapeutic agents from sustained or controlled release formulations (1-4). These include, for the most part, closed systems (e.g. USP methods) in which the accumulated amounts of drug released are measured. Although this may be satisfactory for comparisons of different formulations, the data cannot be extrapolated to represent drug concentrations *in vivo* because of the dynamics of simultaneous drug input and clearance at the site of absorption in a biological system, for example after I.P. administration. An open, flow through method that operates under sink conditions, exemplified by the Langenbucher system which has been designed for dissolution testing has been used mainly for dissolution testing of tablets (5,6) but has problems of filter clogging and pressure build-up (7). Washington and Koosha (8) have described a flow through filtration cell for the study of release from sustained release microspheres.

An open chemostat system which has been routinely used to study the kinetics of interaction between antibiotics and bacteria, allowing simple, convenient determination of the required dose for administration (9), can be modified to study fluid concentrations of drug from sustained release polymeric dosage forms, such as microparticle formulations.

5.2 EXPERIMENTAL

5.2.1 Materials

Poly(L-lactic acid) 100,000 M.W was obtained from Polysciences Inc. (Warrington, PA). Cephalexin monohydrate (CPX) and ciprofloxacin hydrochloride (CFX) were purchased from Sigma Chemical Co., St. Louis, Mo. Diethyl ether and all other chemicals were reagent grade. Demineralized, double-distilled water was used throughout the study.

5.2.2 Preparation of Microspheres

Typically, a sample of CFX or CPX powder was suspended in 10 ml methylene chloride containing 400 mg PLA and stirred (Ikamag stir plate, 400 rpm). Diethyl ether (36 ml) as the nonsolvent was delivered to the stirred solution at a constant rate of 0.2 ml/min using a syringe pump (Sage Instruments, Boston, MA) over a period of three hours. The microspheres which formed were harvested by filtration, washed with 2% polyethylene glycol 400 solution to reduce aggregation and remove drug particles on the surfaces, dried over calcium chloride under vacuum in a desiccator for at least 24 h, then sieve-sized into various fractions.

5.2.3 Determination of Percent Drug Microencapsulated and Drug Loading

Microspheres (10 mg) were added to 5 ml of methylene chloride, a solvent for the polymer but not the drug. Phosphate buffer solution (PBS) (30 ml) was then mixed with the organic solution causing the CFX or CPX particles to dissolve in the aqueous phase. The aqueous phase was separated and the extraction

process repeated two times. Drug concentrations were determined spectrophotometrically at either 328 nm (CFX) or 260 nm (CPX) (Beckman, Model 25 spectrophotometer). Experiments were run in triplicate and the results averaged. The encapsulation efficiency (fraction of initial amount of drug microencapsulated) and drug loading (fraction of microspheres consisting of drug, (% w/w) were determined.

5.2.4 The Chemostat System

A diagram of the chemostat system is shown in Figure 5.1. The chemostat was filled with pH 7.4 PBS as the release medium, then additional medium was pumped at a fixed rate (input) into the vessel (peristaltic pump, Watson Marlow). Simultaneously, medium flowed out of the chemostat at the same constant rate (output) to the waste tank maintaining a constant volume of 120 ml in the chemostat. Water circulation through the walls of the chemostat provided a constant temperature of 37°C. The contents of the chemostat were aerated at a constant air-flow rate of 1 L/min, which provided continuous mixing and ensured homogeneity of the contents.

5.2.5 Determination of Drug Release in the Chemostat

Normally, equivalent weights of either drug powder or drug in microspheres (30 mg of CPX, 7 mg of CFX) were tested. The microspheres were sealed in a polyethylene screen (Spectra/Mesh, 110 μm, Fisher Scientific) to retain them in the chemostat. Medium was pumped into the chemostat at a constant rate of 0.46 ml/min unless otherwise stated. Samples of effluent were withdrawn at specified

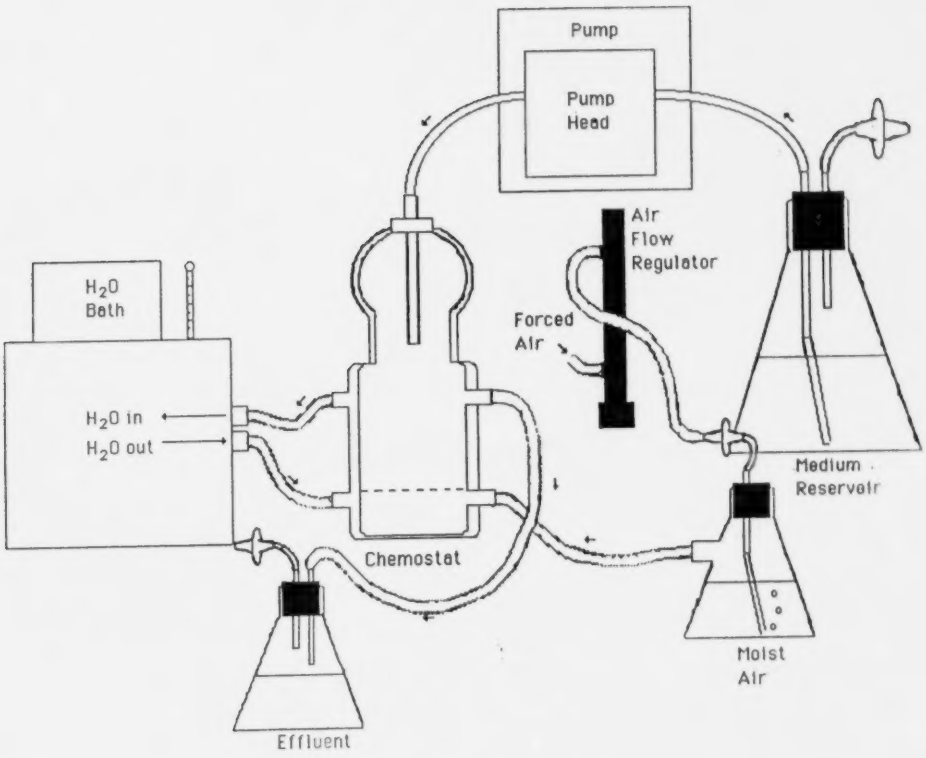

Figure 5.1 Diagram of the chemostat system showing the configuration and structure of the components.

time intervals and analyzed spectrophotometrically, obtaining drug concentrations from a calibration curve. The effects of drug loading, particle size, dose, and release medium delivery rate on the release profiles were examined.

5.3 RESULTS

The microspheres were approximately spherical under the optical microscope and were free-flowing. Separation of a batch of microspheres into various sieve-sized fractions was easily performed. Typically, 42.1%(0.2) (SEM in brackets), 33.4%(0.3), 24.6%(0.4) of a batch of CPX microspheres, and 37.1%(0.3), 24.0%(0.6), 39.0%(0.5) of a batch of CFX microspheres (by weight) were separated into 250-425 μm, 125-250 μm, and < 125 μm sizes, respectively. The percent drug encapsulated for each loading was consistently >90%.

Figure 5.2 and Figure 5.3 illustrate the release profiles and dissolution rate of CPX and CFX, respectively. The solubility of CPX in pH 7.4 PBS was determined to be 40 mg/ml at 37°C (cf. 40 mg/ml, Griffith et al.) (10). The peak concentration of CPX from powder was 225 μg/ml, obtained (from 30 mg) after about 5 min. Release from 33 and 43% w/w PLA microspheres in the open chemostat system resulted in drug concentrations approximately 30 and 60 percent higher after 3 and 6 h, respectively, than that produced from dissolution of the powder, although peak concentrations were lower and occurred at later times. The rate of decrease of concentrations after the peak were approximately equal for the two microsphere loadings.

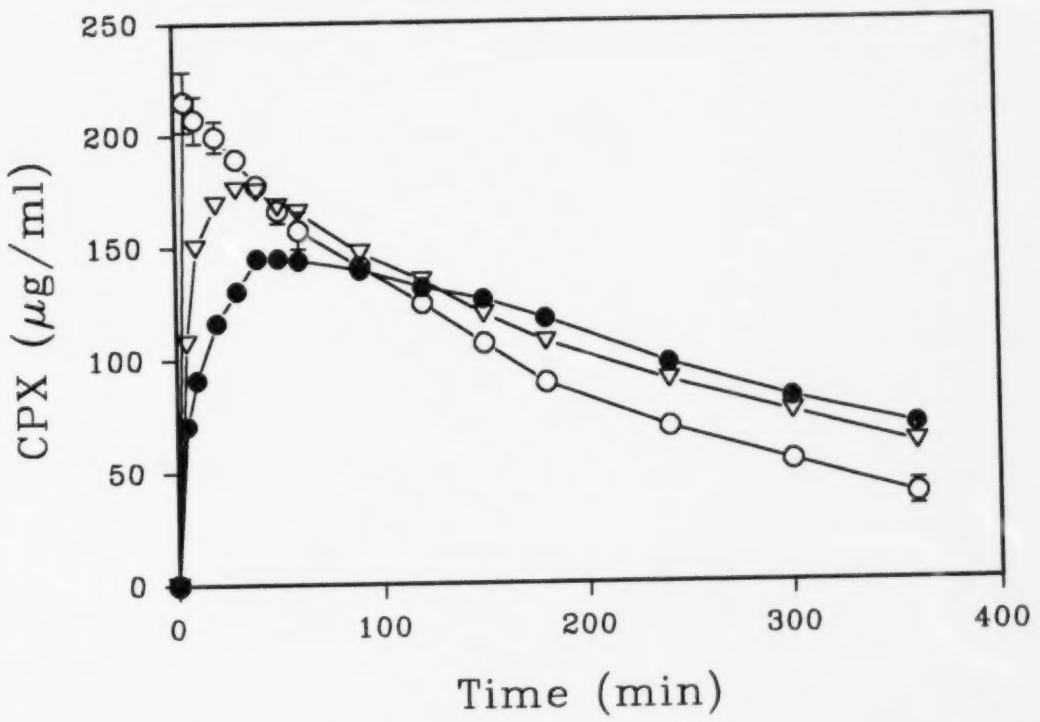

Figure 5.2 CPX release from PLA microspheres in the chemostat at 37° C, pH 7.4 PBS at a dilution rate of 0.46 ml/min as a function of drug loading. (●), 33% w/w; (▽), 43% w/w. (○), dissolution of CPX powder.

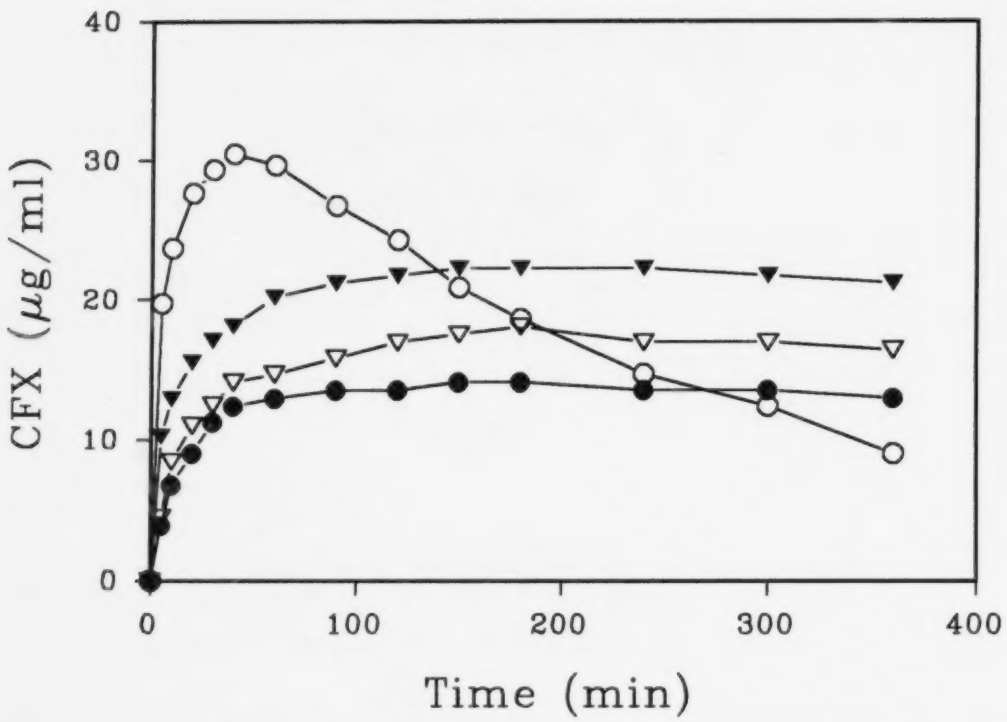

Figure 5.3 CFX release from PLA microspheres in the chemostat at 37° C, pH 7.4 PBS at a dilution rate of 0.46 ml/min as a function of drug loading. (●), 33% w/w; (▽), 50% w/w; (▾), 71% w/w. (○), dissolution of CFX powder.

In contrast, CFX has a solubility of 5 mg/ml at pH 7.4 and its dissolution profile (from 7 mg) indicated a slower rate of dissolution than CPX. The peak concentration of CFX was 30 μg/ml, reached after about 40 min, followed by a gradual decrease in the chemostat concentration reaching 10 μg/ml after 6 h, a 67 percent drop from the peak concentration (cf. 77 percent drop for CPX).

The concentration of CFX obtained from PLA microspheres at a 0.46 ml/min flow rate (Figure 5.3) rose then plateaued at a concentration which was maintained for at least 6 h. The higher the drug loading, the higher the concentration of CFX which was maintained over this time period. Thus, concentrations of 14, 18, and 22 μg/ml of CFX were maintained by microspheres of 33, 50, and 71% w/w drug loading, respectively, in the open chemostat system.

The effect of varying the microsphere mean size on the release of CPX and CFX from PLA microspheres is shown in Figure 5.4 and Figure 5.5, respectively. Microspheres of CPX in the 125-250 μm size range produced a peak CPX concentration very similar to that obtained from the dissolution of CPX powder (about 200 μg/ml) but only after about 35 min (cf. 5 min for the powder). Also, the peak concentration from the microspheres was sustained for about 1 h (the peak concentration of CPX powder was not sustained). In comparison, using 250-425 μm sized PLA microspheres the peak concentration of CPX was 150 μg/ml, reached after about 45 min, which was sustained for approximately 2 h.

CFX powder and microspheres in the 125-250 μm size range also yielded similar CFX peak concentrations, at 30 μg/ml (Figure 5.5) but concentration levels

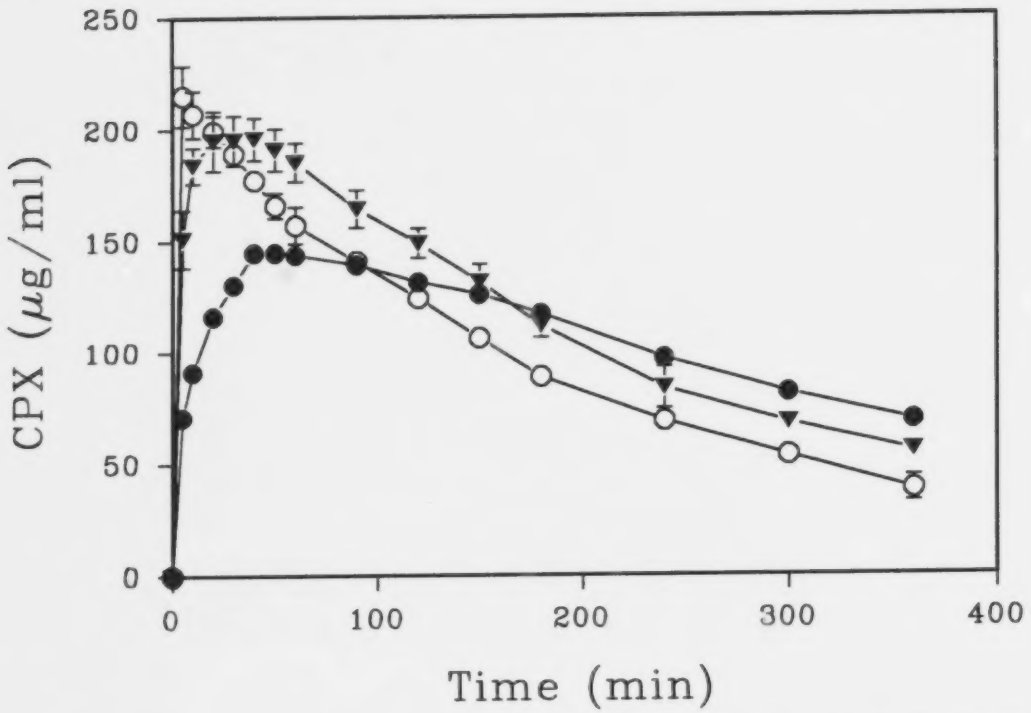

Figure 5.4 CPX release from 33% w/w PLA microspheres in the chemostat at 37° C, pH 7.4 PBS at a dilution rate of 0.46 ml/min as a function of the sieve size range. (●), 250-425 μm; (▾), 125-250 μm; (O), dissolution of CPX powder.

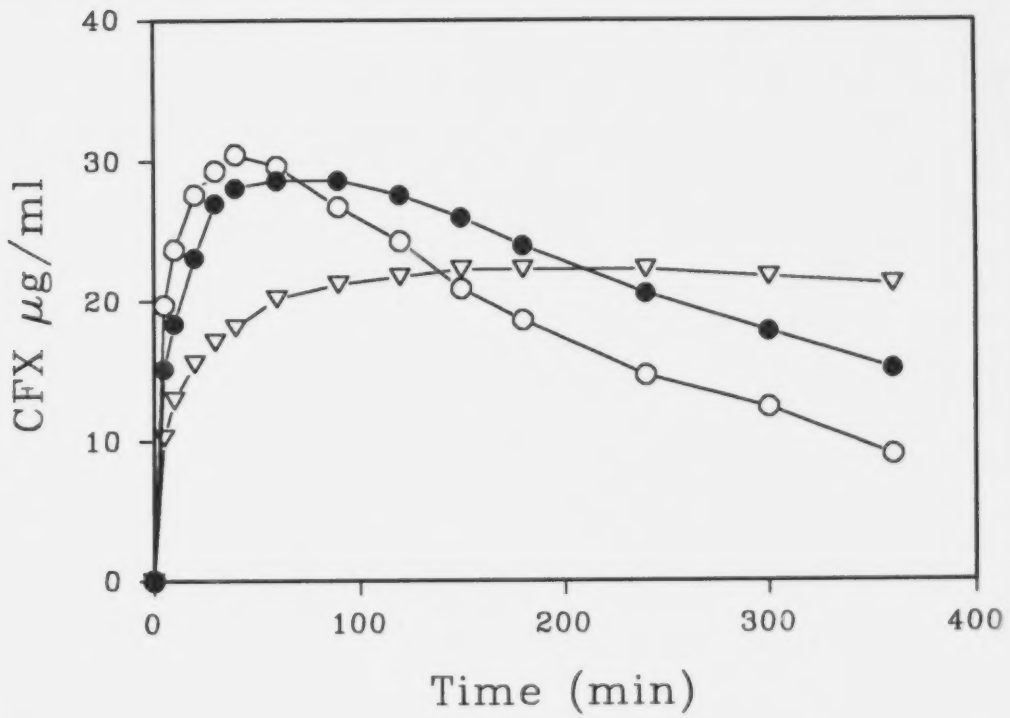

Figure 5.5 CFX release from 71% w/w PLA microspheres in the chemostat at 37° C, pH 7.4 PBS at a dilution rate of 0.46 ml/min as a function of the sieve size range. (●),125-250 μm; (▽), 250-425 μm; (O), dissolution of CFX powder.

were sustained from the microsphere formulation for approximately 1.5 h compared to only 30 min from the powder. In contrast, concentrations of CFX from microspheres in the 250-425 μm size range rose gradually and plateaued at 22 μg/ml after about 2 h, which was maintained in the chemostat for at least 6 h under the flow through conditions.

Figure 5.6 represents the effect of dose on the concentration-time profiles in the open chemostat system. A 7 mg dose or a 10 mg dose of CFX in microspheres resulted in similar profiles, except the peak, steady-state concentration derived from the 10 mg dose was 27 percent higher (28 μg/ml vs 22 μg/ml). In contrast, concentration levels diminished with time to about 5 μg/ml after 6 h for an initial 22 μg/ml solution of CFX in the chemostat.

By adjusting the pump speed, the input/output rate of medium in the chemostat could be varied. It can be seen in Figure 5.7 and Table 5.1 that decreasing the rate from 0.46 ml/min to 0.23 ml/min yielded a higher plateau concentration of CFX of 25 μg/ml but at a later time (2.5 h versus 2 h) whereas increasing the rate to 0.75 ml/min produced a lower plateau concentration of 16 μg/ml, reached after 1.5 h but maintained until about 3 h, after which the concentration gradually decreased to 11 μg/ml after 6 h.

5.4 DISCUSSION

The open chemostat represents a standard first-order kinetic model for the elimination of an initial concentration of drug in solution. However, when a dosage

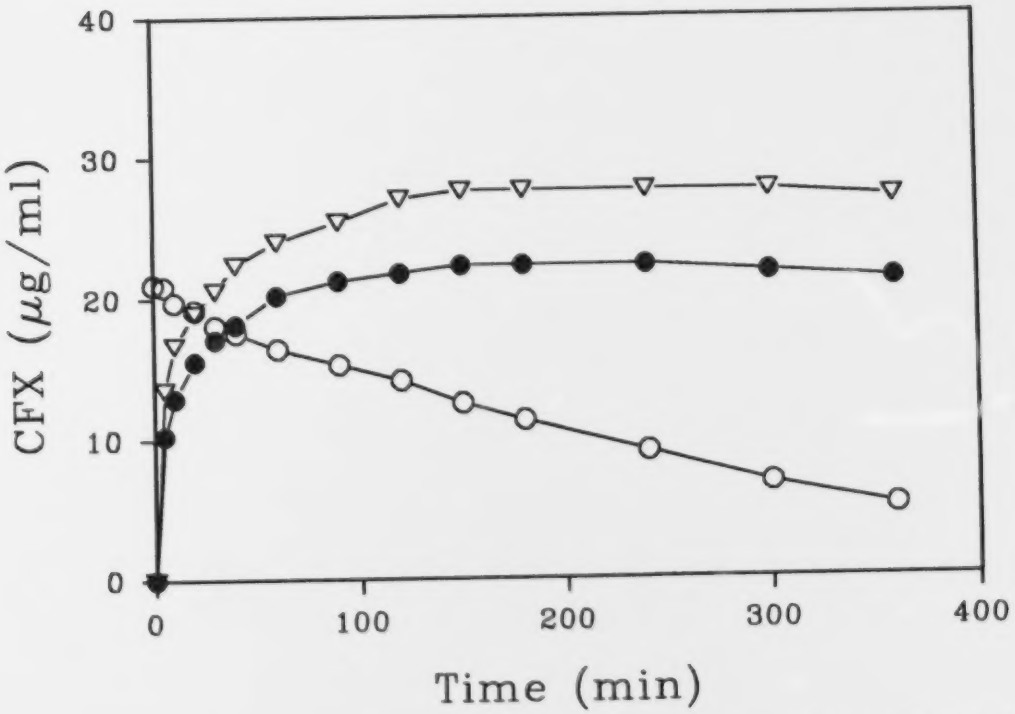

Figure 5.6 CFX release from 71% w/w PLA microspheres in the chemostat at 37° C, pH 7.4 PBS at a dilution rate of 0.46 ml/min as a function of the dose administered. (●), 7 mg; (∇); 10 mg; (O), clearance rate of CFX from a 22 μg/ml solution.

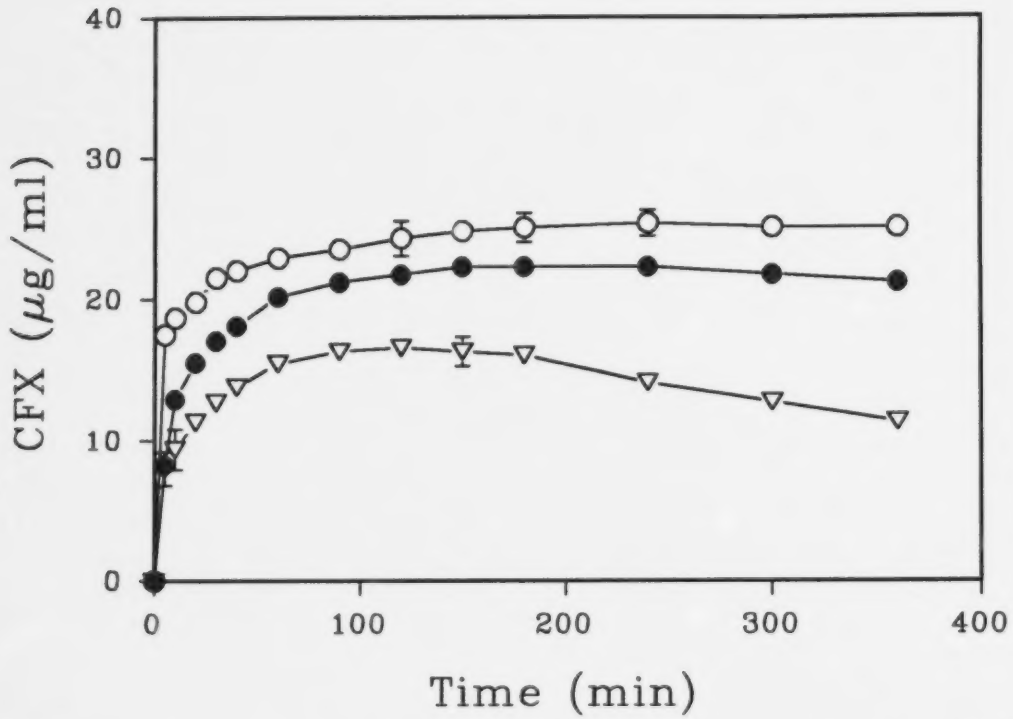

Figure 5.7 CFX release from 71% w/w PLA microspheres in the chemostat at 37° C, pH 7.4 PBS as a function of the dilution rate. (O), 0.23 ml/min; (●), 0.46 ml/min; (▽), 0.75 ml/min.

Table 5.1 Kinetic parameters of CFX release from 71% w/w PLA microspheres in the open chemostat as a function of the flow rate.

Flow Rate (ml/min)	C_{max}[1] (μg/ml)	T_{max}[2] (h)	Duration of C_{max} (h)
0.23	25.0	2.5	4.0
0.46	22.0	2.0	3.0
0.75	16.0	1.5	2.0

[1]maximum CFX concentration in the chemostat after a 7 mg dose of CFX.

[2]time to reach C_{max}.

form is included a new input parameter is introduced due to either a dissolution step or a release phase, depending on the dosage form. In the case of fast dissolution, a peak drug concentration occurs after a short time and then drug concentration decreases according to the kinetics established in the model. Such is the case with CPX because of its relatively high aqueous solubility (Figure 5.2). In the case of CFX, which had a slower dissolution rate (Figure 5.3), the peak occurred at a lower concentration and concentrations fell more gradually thereafter. When a microsphere formulation was used, the rate of drug input was less but concentrations were sustained more for CFX than CPX, correlating with their solubilities. Increasing the drug loading or decreasing the mean microsphere size had the effect of sustaining drug concentrations at higher levels, because of the corresponding higher rates of release.

The dilution rate of 0.46 ml/min in the chemostat was selected because the $t_{½}$ of drug elimination closely approximated the reported biological $t_{½}$ reported for CPX (11) and CFX (12), and is a much slower rate than normally employed in dissolution studies (6). However, different rates of drug removal will likely occur from different sites of administration. Thus, a comparison of the effect of different dilution rates in the chemostat could aid in selecting the formulation having the most favorable release kinetics. For example, 71% w/w microspheres could sustain CFX concentration levels for several hours depending on the dilution rate.

When a sustained release formulation of a drug is injected, for example in the peritoneum, the rate of drug clearance from the peritoneum will be governed by

the rate of release from the formulation and the rate of absorption (and metabolism). Sustained fluid levels of drug are achieved when the formulation has been designed to release drug (input) at a rate that counter-balances the clearance (output) rate. Drug solubility and particle size are important factors in the dissolution process while drug solubility, polymer composition, polymer molecular weight, microparticle size and drug loading are important factors in sustained release. In developing a strategy to determine the optimum dose *in vivo*, an *in vitro* test method which can reveal this for any given formulation would be advantageous. A flow through system such as the open chemostat appears to possess this capability as demonstrated with antibiotic microspheres but it could also be extended to other formulations. The open chemostat does not have problems of clogging, pressure build-up, or inadequate flow rates associated with some flow through systems (7,8). Advantages of the flow through cell method over the USP Paddle or Basket methods for the dissolution of microparticulate systems have been shown (6). Chemostats can be connected in series to facilitate the release studies and the release medium leaving the effluent port of the chemostat can be routed through a spectrophotometer for automatic recording of absorbances. A pH gradient can also be set up to study drug release under changing pH conditions.

The dilution rate of the drug in the chemostat can be adjusted to simulate the clearance of drug *in vivo* which forms a basis for comparison of formulations. The intraperitoneal route of administration is particularly appropriate for formulations

characterized by open chemostat studies because local concentrations of antibiotic maintained above the MIC for a sufficiently long period of time are required to treat infections in the peritoneum. In this case, selection of the microsphere size and loading that provides sustained concentrations of antibiotic can be made from chemostat studies once the pharmacokinetics of the drug in the peritoneum are known. It is then a simple matter of adjusting the flow rate in the chemostat and developing formulations which produce sustained concentrations of drug at a level appropriate for effective therapeutic treatment. It is suggested that the use of the chemostat in developing new sustained release formulations could also reduce the requirement of animals to define the relevant parameters.

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CHAPTER 6

EFFECTIVENESS OF CIPROFLOXACIN MICROSPHERES IN ERADICATING BACTERIAL BIOFILM

6.1 INTRODUCTION

Peritoneal dialysis has been recognized as a major form of therapy for chronic renal failure, but peritonitis associated with this procedure is a limiting factor (1,2). Administration of antibiotics in peritoneal dialysate solution is often not very effective since recurrent peritonitis due to bacterial biofilm often leads to complications.

Bacterial biofilm consists of microbes that are able to attach firmly to solid surfaces, such as catheters or prosthetic devices and produce a glycocalyx matrix, inside of which microcolonies develop. These microcolonies coalesce to form biofilm (3-5). Infections due to biofilm on the devices are of great concern to the clinician and microscopic examination of infected devices removed from patients has conclusively revealed that both gram-positive and gram-negative bacteria have been implicated (3,5-7). The establishment of biofilm, especially aged biofilm, on the surfaces of medical devices such as catheters in vivo, offers invading pathogens survival advantages and, consequently, host immune defences are less effective (4,8). At the same time there is increased resistance to antibiotics (9-11).

The principal organisms responsible for peritonitis are *S. epidermidis* and *S. aureus*, although the range of pathogens is broad and includes *P. aeruginosa* and the Enterobacteriaceae (14-16). Ciprofloxacin (CFX) is a fluoroquinolone antibiotic with activity against both gram-positive and gram-negative bacteria *in vitro* (17). Frequent administration of CFX intraperitoneally to treat peritonitis in CAPD has not halted recurrent peritonitis (15,16) and this may be due to bacterial biofilm

which was not completely eradicated. In peritonitis, the peritoneal membrane has an increased permeability and the half-life of the antibiotic in the peritoneum is lower than that in patients without peritonitis due to rapid transperitoneal absorption (12). On the other hand, most orally or systemically-administered antibiotics permeate the peritoneum poorly (13).

An open chemostat system has been developed to study the kinetic interaction of antibiotics against bacteria in biofilm (9-11). Such a system represents a one-compartment, kinetic model of the peritoneum which can be used to study the pharmacokinetics of drugs within the peritoneum. The effectiveness of sustained release poly(L-lactic acid) (PLA) microspheres of CFX against *S. aureus*, and *P. aeruginosa* aged biofilm on silicone tubing normally used in catheters has been studied. PLA was selected because its biodegradability and histocompatibility have been well established from its uses in surgical suitures, grafts, implants, and various prosthetic devices (18-20).

6.2 EXPERIMENTAL

6.2.1 Materials

Iron-depleted tryptic soy broth (TSB-Fe; Difco Laboratories, Detroit, Mich.), 2,2-dipyridyl (Terochem Laboratories, Edmonton, Alberta).

Bacteria. Strains of mucoid *S. aureus* R627 and *P. aeruginosa* M579 (Department of Microbiology, University of Alberta, Edmonton, Alberta) were maintained as freeze-dried cultures and recovered by growth on nutrient agar plates. After

overnight incubation at 37°C, the growth from the plates was inoculated into 100 ml of iron-depleted tryptic soy broth (TSB-Fe) medium.

6.2.2 Methods

The open chemostat system described by Anwar et al. (9-11) was adopted for these studies. *S. aureus* R627 or *P. aeruginosa* M579 bacteria were grown in a chemostat containing iron-depleted tryptic soy broth (TSB-FE) to which 2,2-dipyridyl (final concentration, 25 μg/ml) had been added as a chelating agent. Chemostats containing 100 pieces each of 1-cm long Masterflex silicone tubing (size 16) (Cole Parmer Instrument Ltd., Chicago, IL) were autoclaved at 121°C for 20 min. Subsequently, the chemostat was inoculated with 5 ml of *S. aureus* or *P. aeruginosa* culture grown to mid-exponential phase in TSB-FE and maintained at 37°C for 7 days for cell attachment and biofilm formation. Afterwards, the TSB-FE medium was delivered to the chemostat at a constant rate (input) of 0.23 ml/min using a multichannel peristaltic pump and the contents simultaneously flowed out at the same rate (output). Under these conditions, the volume of each chemostat was maintained at 100 ml for the duration of the experiment. Air was bubbled through the mixture which served to aerate as well as mix the contents.

On day 7 after inoculation, viable counts of planktonic cells were determined from serially-diluted samples incubated on nutrient agar plates (Difco Laboratories, Detroit, MI). The number of biofilm cells that adhered to the pieces of silicone tubing was determined by removing the tubing, gently washing it three times with 10 ml of phosphate-buffered saline (PBS) to remove non-adhering cells, placing

it in 1 ml of PBS, vortex-mixing for 3 min, preparing serially-diluted samples, inoculating nutrient agar plates with the contents, incubating at 37°C, then counting the cells (21). The efficiency of removal of biofilm cells from the tubing was checked by repeating this procedure until the number of cells remained constant. Usually, 99% of the adhering biofilm cells were removed during the first vortex-mixing step in agreement with previous observations(9,21,22). The population of biofilm cells by day 7 was standardized at 10^8 colony-forming cells (CFU) per cm of tubing.

6.2.3 Method of Preparation of Microspheres

Microspheres were prepared using a coacervation procedure. Either 200, 400, or 1000 mg of CFX (< 250 μm) were dispersed in 6 ml of methylene chloride by probe sonication (Heat Systems, Model W-375, Plainview, N.Y.) then transferred to a round bottom flask containing 6 ml of a solution of PLA in methylene chloride to make a final concentration of 40 mg/ml of PLA (100,000 M.W.). Magnetic stirring was carried out at 400 rpm (Ikamag stir plate). Diethyl ether (36 ml) was added dropwise at a constant rate of 0.2 ml/min using a syringe pump (Sage Instruments, Boston, MA) for 3 h. The microspheres which formed were harvested by filtration, (Whatman No. 5, nominal porosity 2.5 μm), washed with water then vacuum-dried over $CaCl_2$ in a desiccator. The dried material was subsequently dispersed in diethyl ether to break up clumps, then filtered and dried. The product used was a sieve-sized fraction of 40/60 mesh (250-425 μm).

6.2.4 Determination of the Interaction of Planktonic and Biofilm Cells with CFX

On day 7 after removal of bacterial samples for viable count determination, microspheres were sealed in a nylon screen (Spectrum, Houston, TX) previously sterilized, and placed in the chemostat (23). Subsequently, antibiotic-free medium was pumped into the chemostat at a rate of 0.23 ml/min. Samples were removed at pre-determined time intervals and viable counts of either planktonic or biofilm cells were performed on nutrient agar plates.

CFX concentrations in the chemostat were determined at various time intervals by centrifuging (5,000 x g, 15 min) 1 ml of the effluent, removing the supernatant, then analyzing diluted samples spectrophotometrically at 328 nm. Concentrations of CFX were obtained from a calibration curve. Microspheres were added to the chemostat once each day for four days in all cases. When it was observed that bacteria were killed, fresh TSB-FE was continuously pumped into the chemostat at the same rate for another 3 days to detect the possible re-growth of the organisms.

The effects of CFX loading and dose of microspheres on the killing of both planktonic and biofilm cells of *S. aureus* and *P. aeruginosa* were compared with that of the free drug. The results have been given as averages of duplicate determinations.

6.3 RESULTS

6.3.1 Effect of Microsphere Loading on CFX Ryelease and S. aureus Biofilm Eradication

Typical release profiles of CFX at loadings of 33, 50, and 71% w/w in the presence of biofilm and planktonic cells of *S. aureus* are shown in Figure 6.1. It can be seen that CFX concentrations increase rapidly, reaching a peak concentration, C_{max}, which is sustained for a period of time, then concentrations decrease at a rate determined by the balance of input rate (release rate) and output rate (dilution rate) in the chemostat. The higher the loading, the higher the C_{max} attained, the longer the duration of the C_{max} and the greater the AUC. As a result, a loading of 71% w/w CFX maintained higher concentrations in the chemostat for 24 h than at lower loadings. A quantitative comparison of the various kinetic parameters of the three formulations is given in Table 6.1. Microspheres of 71% w/w loading sustained a concentration of 37.2 μg/ml CFX in the chemostat for 7 h, falling to only about 15 μg/ml after 24 h of continual dilution, a concentration considerably above the MIC of CFX for these bacteria (24).

The rates of killing of planktonic and biofilm cells in the chemostats under conditions of CFX release from microspheres as a function of loading are depicted in Figure 6.2. It is apparent that at 71% w/w loading, microspheres effectively reduced cell populations to almost zero after 3 days, following dosing every 24 h after the initial dose (Figure 6.2A). In comparison, the cell populations diminished (at a slower rate) to 10^{-4} - 10^{-5} CFU/ml at 50% w/w loading and 10^{6} CFU/ml at 33%

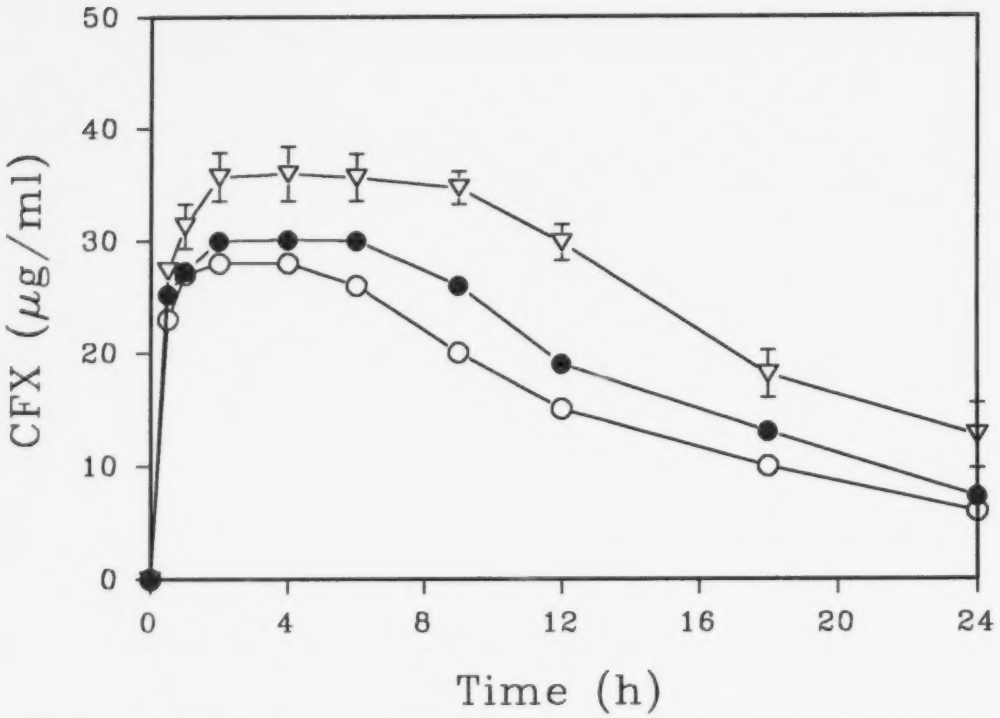

Figure 6.1 Release of CFX from PLA microspheres in the open chemostat in the presence of *S. aureus* planktonic and biofilm cells as a function of loading. (○), 33; (●), 50; (▽), 71% w/w.

Table 6.1 Kinetics of release of CFX from PLA microspheres in the open chemostat in the presence of *S. aureus* biofilm and planktonic cells as a function of loading.

CFX Conc. (% w/w)	C_{max}[1] (μg/ml)	T_{max}[2] (h)	Duration of C_{max} (h)	C_{24h}[3] (μg/ml)	AUC[4] (μg.h/ml)
33	25.3(2.5)	1.0(0.0)	4.0(0.0)	8.3(3.2)	485
50	31.6(1.6)	2.0(0.0)	5.0(0.5)	8.8(1.4)	584
71	37.2(2.2)	2.0(0.0)	8.0(0.3)	14.7(3.0)	814

Standard error of the mean (SEM) shown in brackets. All points within the SEM of the C_{max} were included in the duration of C_{max}.

[1]average daily maximum CFX concentration after administering a 7 mg dose of CFX each day for 4 days.

[2]time to reach the C_{max}.

[3]average daily minimum CFX concentration over 4 days.

[4]area under the concentration-time curves, calculated using linear trapezoidal method of program Lagran.

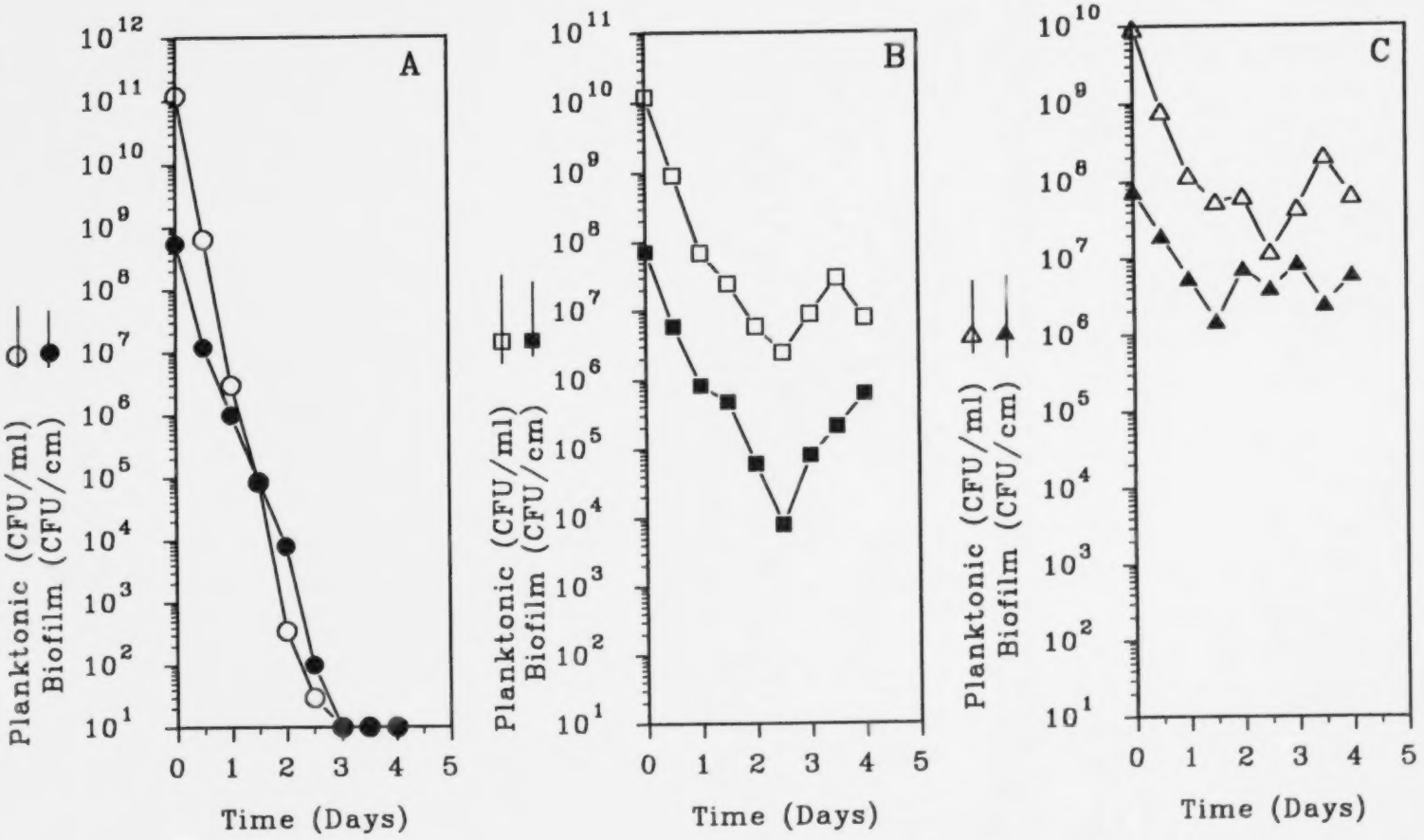

Figure 6.2 The rate of killing of planktonic and biofilm cells of *S. aureus* by CFX released from PLA microspheres as a function of loading. **A**. 71; **B.** 50; **C**. 33% w/w. The chemostat was dosed every 24 h after the initial dose.

w/w loading of CFX after 1-2 days, then reversed and increased significantly thereafter (Figure 6.2C).

6.3.2 Release Profiles and S. aureus or P. aeruginosa Biofilm Eradication as a Function of the Dose of CFX

Increasing the dose of 71% w/w CFX PLA microspheres in the chemostat increased C_{max}, the duration of C_{max} and the AUC over a 24 h period (Figure 6.3). At a 7 or 10 mg equivalent dose of CFX, concentrations were maintained above 15 μg/ml for 24 h and as high as 37 μg/ml for 7-8 h (Table 6.2). This was adequate to reduce planktonic and biofilm cell populations to near zero after 2.5-3 days (Figure 6.4B and 6.4C). In contrast, a 3.5 mg dose of either free CFX or CFX microspheres, or a 5 mg dose of CFX in microspheres reduced cell populations to only 10^6 CFU/ml for 1.5 days, after which the number of cells began to increase. (Figure 6.4A).

Studies with gram-negative *P. aeruginosa* bacteria produced the results shown in Figure 6.5 and Table 6.3. Although the kinetic parameters, C_{max} and C_{24h} were slightly lower than in the presence of *S. aureus* (Table 6.2), a 7 mg dose of CFX microspheres was as effective (Figure 6.5A), whereas a lower dose or 3.5 mg of free CFX were ineffective (Figure 6.5B and 6.5C). Previously, it had been shown that administration of 7 mg of free CFX was ineffective against *P. aeruginosa* biofilm (A. Anwar, unpublished data).

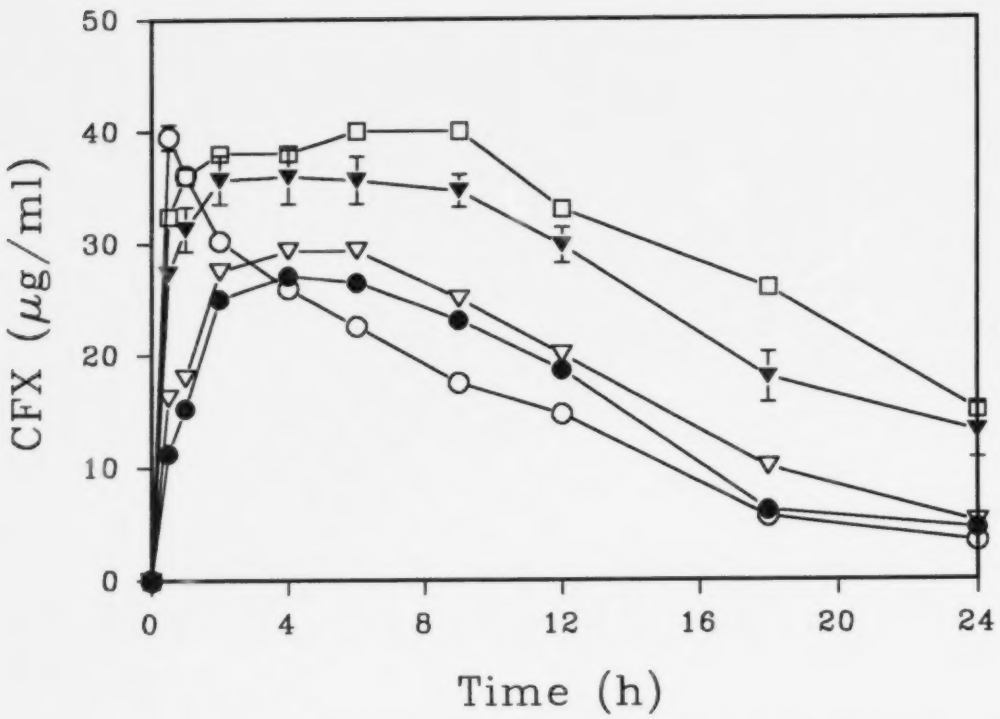

Figure 6.3 Release of CFX from 71% w/w PLA microspheres in the presence of *S. aureus* planktonic and biofilm cells as a function of the dose of CFX. (O), 3.5 mg of free CFX ; (●), 3.5 mg equiv. wt.; (▽), 5 mg equiv. wt.; (▾), 7 mg equiv. wt.; (□), 10 mg equiv. wt.. The dissolution of free CFX is shown for comparison.

Table 6.2 Kinetics of release of CFX from 71% w/w PLA microspheres in the open chemostat containing *S. aureus* biofilm and planktonic cells as a function of the dose

CFX Dose (mg)	C_{max}[1] (μg/ml)	T_{max}[2] (h)	Duration of C_{max} (h)	C_{24h}[3] (μg/ml)	AUC[4] (μg.h/ml)
3.5[5]	39.8(2.4)	0.5(0.0)	0.0(0.0)	2.5(1.0)	382
3.5	26.6(1.1)	2.0(0.0)	3.0(0.4)	5.6(1.4)	404
5.0	30.3(2.5)	2.0(0.0)	4.8(0.9)	9.4(3.3)	480
7.0	37.2(2.2)	2.0(0.0)	7.3(0.3)	14.7(3.0)	814
10.0	38.3(2.0)	2.0(0.0)	8.5(0.5)	15.3(2.1)	1062

[5]Free CFX. See Table 6.1 for definitions of terms.

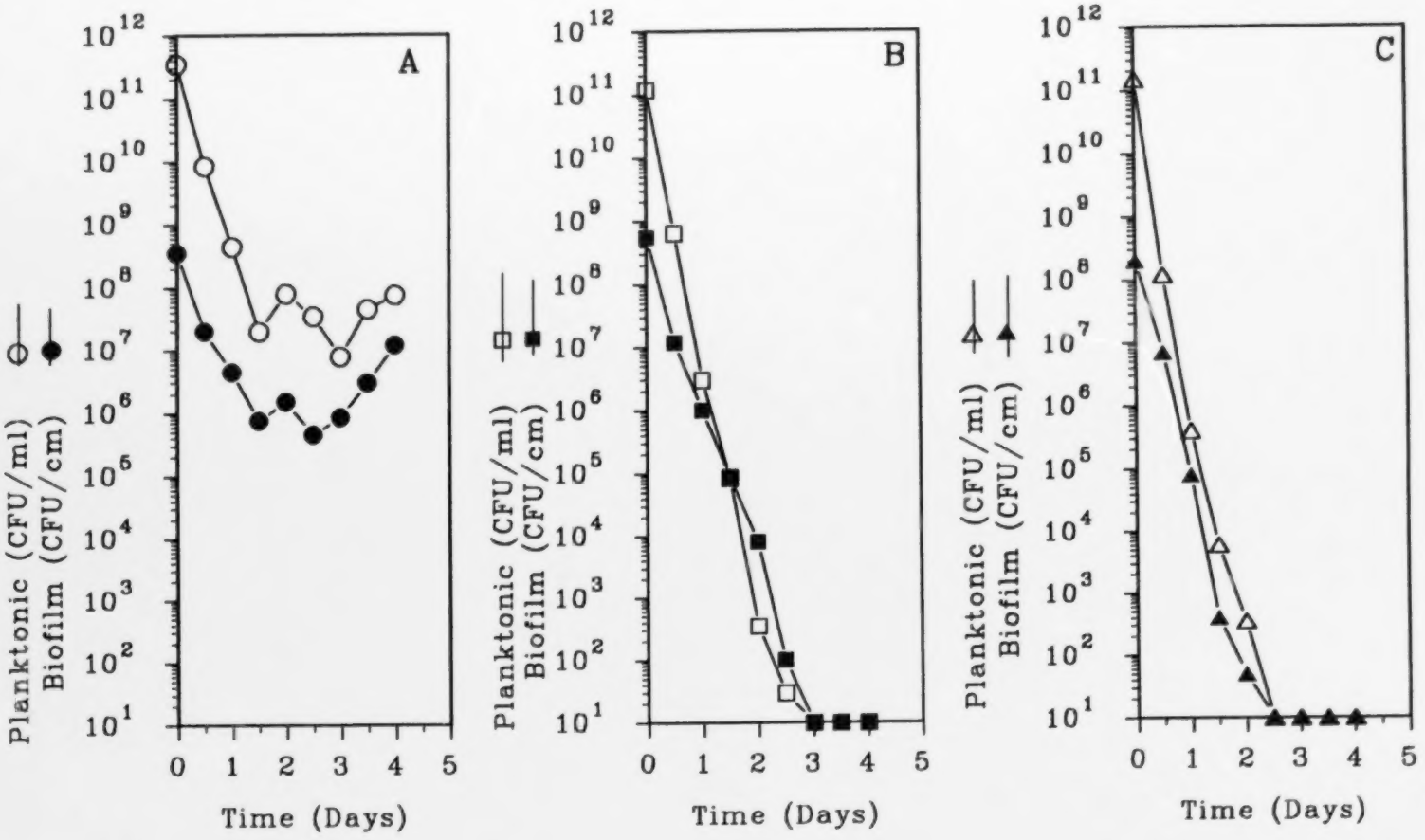

Figure 6.4 The rate of killing of planktonic and biofilm cells of *S. aureus* by CFX released from 71% w/w PLA microspheres as a function of the dose. **A**. 3.5 or 5 mg equiv. wt. of CFX in microspheres or 3.5 mg free CFX; **B**. 7 mg equiv. wt. of CFX; **C**. 10 mg equiv. wt. of CFX.

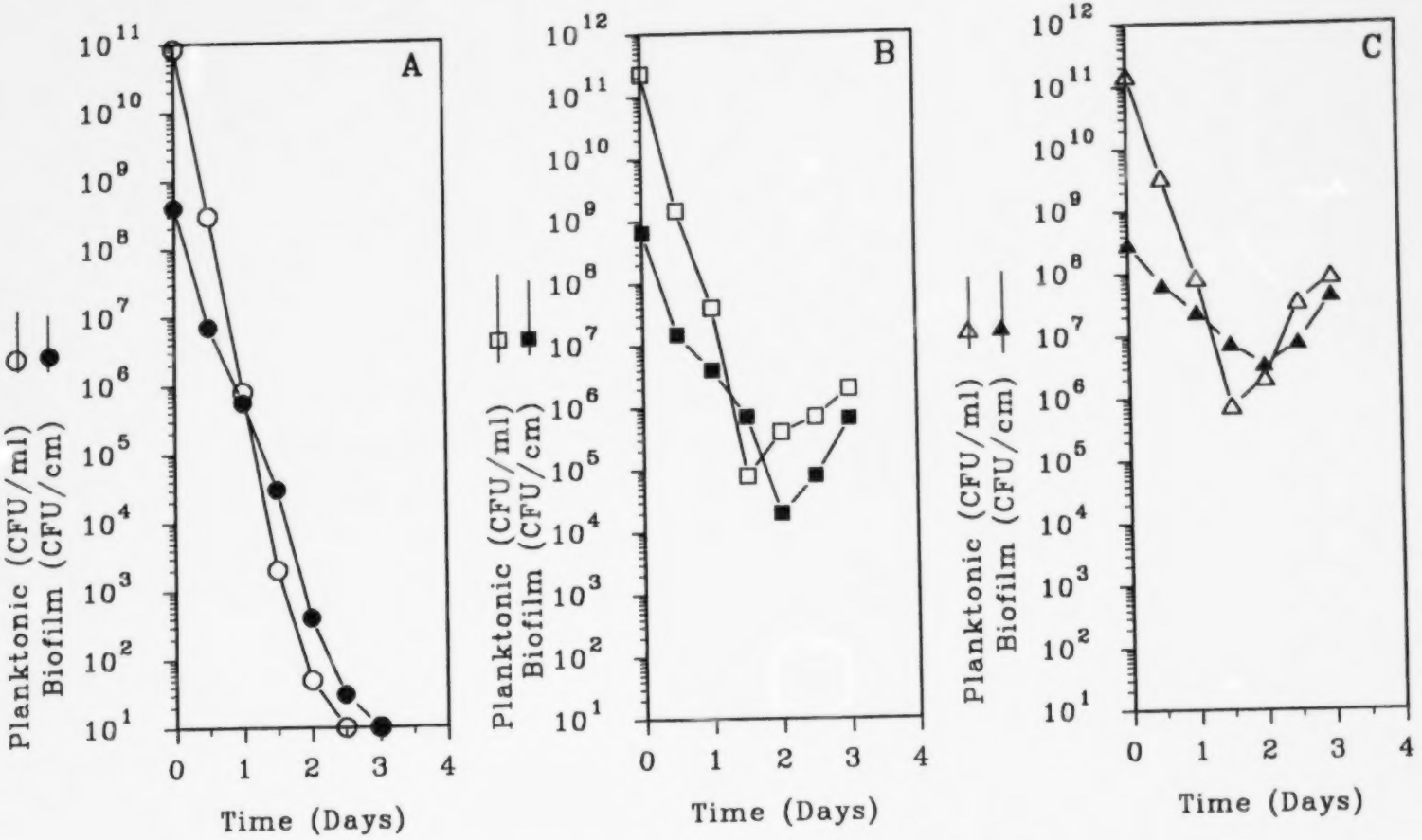

Figure 6.5 The rate of killing of planktonic and biofilm cells of *P. aeruginosa* by CFX released from 71% w/w PLA microspheres as a function of the dose. **A**. 7 mg equiv. wt of CFX; **B**. 5 mg equiv. wt. of CFX; **C**. 3.5 mg free CFX.

Table 6.3 Kinetics of release of CFX from 71% w/w PLA microspheres in the open chemostat containing *P. aeruginosa* biofilm and planktonic cells as a function of the dose.

CFX dose (mg)	C_{max}[1] (μg/ml)	T_{max}[2] (h)	Duration of C_{max} (h)	C_{24h}[3] (μg/ml)	AUC[4] (μg.h/ml)
3.5[5]	38.4(2.3)	0.5(0.0)	0.0(0.0)	0.0(0.0)	380
5.0	26.6(1.3)	2.0(0.0)	3.0(0.0)	0.6(0.1)	397
7.0	34.6(2.8)	2.0(0.0)	6.0(0.0)	6.2(0.9)	741

[5]Free CFX. See Table 6.1 for definition of terms.

6.4 DISCUSSION

The effectiveness of antibiotics against pathogens has been shown to be a function of the dose and the physiology of the pathogens, which is influenced by the availability of certain nutrients in the growth medium (3,10,25). When bacteria are permitted to grow and attach to medical devices, such as an in-dwelling catheter, the bacteria become further resistant to treatment due to biofilm formation. Old (aged) biofilm cells of *P. aeruginosa* were reported to be unharmed upon exposure to 20 μg/ml of tobramycin for more than 5 h and only 50 percent of the viable cells were eliminated after exposure to 50 μg/ml. When aged biofilm cells were removed and became planktonic cells, the MIC was found to be 1 μg/ml (26). Since these concentrations of antibiotic are relatively high either a more potent antibiotic should be used or, as considered here, the sustained release of higher concentrations of antibiotic has been shown to be effective.

The flow rate of medium through the open chemostat determines the rate of clearance of a drug solution in the chemostat. The concentration of drug in the chemostat at any given time is determined by the dilution rate at constant volume, which for an initial concentration, C, at t = 0 falls until all of the drug has been washed from the system, and is described by first order kinetics. However, if the chemostat is infused with drug, the concentration of drug at any time will be a function of the infusion rate and the dilution rate. The infusion rate of CFX in this instance was provided by the kinetics of release of CFX from the microspheres, which has been previously shown to be controlled by formulation (23). The flow

rate of 0.23 ml/min used in the chemostat corresponds to a biological elimination $t_{½}$ of about 5 h for CFX (17). Hence, the concentration-time profiles shown in Figure 6.1 and 6.3 should approximate a situation found *in vivo* after administration of a formulation I.P. for the treatment of localized bacterial infections.

A correlation was found between sustained CFX concentrations as a function of dose or microsphere loading and the killing of planktonic cells and the eradication of biofilm of either *S. aureus* or *P. aeruginosa*. Both C_{max} and duration of C_{max} are important parameters of the minimum requirements for treatment because these determine the AUC over the observation period. Thus, an AUC of about 800 μg.h/ml appeared to meet the requirement and this was achieved under chemostat conditions with at least a 7 mg dose of 71% w/w microspheres.

The exact composition and behavior of glycocalyx produced by biofilm cells is not clearly known. It has been suggested, however, that binding of antibiotic to the glycocalyx may impede its penetration (3,27,28), and the matrix of the biofilm changes and becomes more resistant in response to exposure to antibiotic (3,29). The administration of a biofilm eradicating concentration (BEC) greater than the MIC of planktonic cells has been stressed (22) in order to prevent bacterial biofilm cells from expressing antibiotic-resistant factors. It is concluded from this study that a sustained BEC of CFX shows promise for the treatment of device-associated bacteria biofilm infections.

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CHAPTER 7

EFFECTIVENESS OF SUSTAINED RELEASE CIPROFLOXACIN MICROSPHERES AGAINST DEVICE-ASSOCIATED PS. AERUGINOSA BIOFILM INFECTION IN THE RABBIT PERITONEAL MODEL

7.1 INTRODUCTION

Medical or prosthetic devices have been implanted in millions of human subjects to save lives or to improve the quality of life, and the application may be either for short or long term (1,2). The implantation of several medical devices such as aortic interposition grafts, peritoneovenous shunts, ventriculoperitoneal shunts, t-tubes and peritoneal dialysis catheters within the peritoneal cavity results in infections (3). Although most implant-related infections are caused by gram-positive bacteria, infections due to gram-negative bacteria are potentially more serious (3,4). These are complicated by the presence of bacterial biofilm - microbes that are able to attach firmly to solid surfaces such as catheters or prosthetic devices, and produce a glycocalyx matrix, inside of which microcolonies coalesce and thrive (1,5,6). This has been confirmed by microscopic examination of infected devices removed from patients (1,7). The establishment of biofilm on the surfaces of medical devices, such as catheters *in vivo*, offers invading pathogens survival advantages and consequently, host immune defences are less effective (5,8). At the same time there is increased resistance to antibiotics (9-11).

Peritoneal dialysis has been recognized as a major form of therapy for chronic renal failure, but peritonitis associated with this procedure is a limiting factor, especially recurrent peritonitis (12,13). Many cases of these chronic infections have been attributed to biofilms associated with peritoneal catheters (14-16). The principal pathogens responsible for continuous ambulatory peritoneal dialysis (CAPD) peritonitis are *S. epidermidis*, *S. aureus*, *E. coli* and *P. aeruginosa* (17-19).

The current mode of treatment of CAPD peritonitis is by administration of antibiotics in peritoneal dialysate solution (20-24). The elimination half-life of most antibiotics in the peritoneum is shorter than in the serum due to rapid transperitoneal absorption. In peritonitis, the peritoneal membrane has an increased permeability and the half-life of the antibiotic in the peritoneum is further reduced compared to patients without peritonitis (20-24). Although intraperitoneal administration of antibiotics including ciprofloxacin HCl (CFX) appears to be effective, the emergence of resistant strains and recurrent peritonitis are still problems (21-23). This may be attributed to the occurrence of bacterial biofilm on peritoneal catheters due to a subtherapeutic concentration of antibiotic in the peritoneum towards the end of the dwell time of the dialysate. This problem could be overcome by the administration of a sustained release antibiotic formulation, such as a microparticle which will be able to deliver the antibiotic continuously during the dwell time of the peritoneal dialysate. The formulation of CFX in biodegradable polymeric microspheres has been reported previously (25) and the effectiveness of the microspheres in eradicating bacterial biofilms of *P. aeruginosa* and *S. aureus* in the open chemostat has been demonstrated (Chapter 6). CFX was chosen for this study because it is very active against both gram-positive and gram-negative bacteria *in vitro* (26) and it has been administered in peritoneal dialysate to treat CAPD peritonitis in the clinic, (21-23). The aims of this study were (a) to determine the pharmacokinetics of microencapsulated and free unencapsulated CFX in a rabbit peritoneal model, and (b) to determine the

effectiveness of a sustained release CFX microsphere formulation in treating *P. aeruginosa* biofilm growing on an implanted "lifesaver device" as well as the catheter in the peritoneum of the rabbit.

7.2 EXPERMENTAL

7.2.1 Materials.

Disks of Silastic subdermal implant devices and CAPD catheters with dacron cuffs (9 FR ID) were purchased from Dow Corning Corp., Medical Products, Midland, MI, USA. Adult female New Zealand white rabbits (Vandermeer C., Sherwood Park, Alberta, Canada) weighing 3 - 3.5 kg were used in all experiments. Mucoid *P. aeruginosa* PAO-1 was used for precolonization of the devices.

7.2.2 Pharmacokinetics of Microencapsulated and Free CFX

Six adult female New Zealand white (NZW) rabbits were placed under halothane anaesthesia in the left lateral recumbency. The entire area from the dorsal midline to the ventral midline, and from the point of the hip to the mid thorax were shaved and given three surgical scrubs with betadine solution and spray (Purdue Fredric Co., Toronto, Ontario, Canada). In addition, the right jugular vein and dorsal midline area were shaved and surgically prepared. A mid-line incision was made and a CAPD catheter was placed in the abdomen with the external segment tunnelled subcutaneously to the dorsal midline. The distal end of the catheter was then exteriorized and attached to an injection adaptor. The right

jugular vein was exposed and cannulated with an 5F intravenous catheter. A skin incision was made over the dorsal midline of the back in order to facilitate the subcutaneous tunnelling of the iv catheter. A three-way stop cock and a butterfly bandage was sutured securely in place. An intravenous drip was connected and intravenous fluid (5% dextrose + no. 75 electrolyte, Baxter, Deerfield Il) was administered to ensure iv catheter patency. All surgical procedures and the handling and care of animals were according to the guidelines for the use of experimental animals of the Canadian Council on Animal Care (CACC). The animals were allowed to recover and placed in individual rabbit restraining cages.

The rabbits initially received 250 ml of peritoneal dialysate (Dianeal, Baxter, Deerfield Il) then were divided into 3 groups of 2 rabbits. At the end of 12 h, the rabbits received an additional 250 ml of peritoneal dialysate. In addition, groups 1, 2 and 3 received either 9 mg of blank microspheres, 10 mg of free CFX,, or 20 mg of CFX in microspheres in peritoneal dialysate, respectively. Two milliliters of peritoneal fluid were withdrawn through the peritoneal catheter at times 0, 0.5, 1, 2, 4, 6, 8, 10 and 12 h. Subsequently, the samples were centrifuged, then stored at -20°C until required for further analysis. A 2 ml-sample of blood was also withdrawn via the jugular vein at approximately the same times, placed in serum separator tubes (Corvac, Sherwood Medical, St. Louis, MO) and centrifuged. The recovered serum was removed and stored at -20°C.

Analysis of CFX in peritoneal dialysate and serum - A serum sample (0.5 ml) was combined with 0.5 ml acetonitrile to precipitate protein and centrifuged (5,000 x g,

5 min). A 0.7 ml aliquot of supernatant was evaporated to dryness and reconstituted with 0.5 ml of phosphate buffer (pH 7.4) containing 5 μg/ml of quinine sulphate (as the HPLC internal standard). Diluted samples of peritoneal dialysate containing internal standard were also prepared. Analysis of CFX was carried out by reverse phase HPLC. Conditions of analysis included a C_{18} Nova-Pak column (Waters-Millipore Corp., Milford, MA.), a Waters 501 pump connected to a WISP automatic sampler, and a Waters 740 scanning fluorescence detector. Excitation and emission wavelengths were set at 280 and 455 nm, respectively. The mobile phase consisted of acetonitrile:33.5mM phosphate buffer (16.5:83.5 v/v) adjusted to pH 3.0 with glacial acetic acid. Retention times were 4.3 and 7.9 min for ciprofloxacin and quinine sulphate, respectively. The sensitivity of the assay was 0.01 μg/ml and linear regression analyses of standard calibration curves yielded a correlation of 0.999 over the range 0.02 to 10 μg/ml. The daily coefficient of variation was < 4%.

7.2.3 Procedure for Testing CFX Formulations Against Severe Device-Related Biofilm Infections

Lifesaver implant devices - The lifesaver devices were constructed according to Ward et al. (27). Briefly, 1.5 cm-diameter disks of Silastic subdermal implant material (Dow Corning) were attached to both sides of six teflon support disks of the same diameter. The support disks were then suspended on a 4.0 cm Teflon rod separated by 2 mm Teflon spacers. The total surface area of the Silastic material for each device was 32 cm^2. The devices were sterilized with ethylene

oxide for 4 h prior to colonization.

Pre-colonization of implant devices - Fresh, sterilized nutrient broth (50 ml) was inoculated with a 2 % active inoculum of *P. aeruginosa* PAO-1. Sterile lifesaver devices were pre-colonized in each broth culture for 48 h at 37°C in a shaker incubator. A total of 6 lifesaver devices were pre-colonized. After the incubation period, the lifesaver devices were aseptically removed from the broth cultures and rinsed with sterile phosphate buffered saline (PBS) to remove any planktonic bacteria.

Implantation of lifesaver device - After pre-colonization, one disk was aseptically removed from each lifesaver device and the number of biofilm cells determined. During surgery, the device was placed in the peritoneal cavity of a rabbit adjacent to the tip of the CAPD catheter. The device was fixed to the abdominal peritoneal wall with a single 2-0 polyproplyene suture (Prolene, Ethicon, Peterbourough Ontario, Canada). After recovery, all the rabbits were placed in individual cages and monitored daily for food and water consumption, urination, defecation and incision site abnormalities.

Treatment with microencapsulated or free CFX - Initially (run 1), 6 rabbits divided into 3 groups of 2 received either 9 mg blank microspheres, 10 mg free CFX, or 20 mg CFX-loaded PLA microspheres, respectively. Subsequently (run 2), 6 rabbits divided into 2 groups of 3 received either 10 mg free CFX or 20 mg CFX-loaded PLA microspheres, respectively. In each case, the formulation was administered in the dialysate. Twenty-four hours after implantation of a lifesaver

device during run 1, each rabbit received 250 ml of peritoneal dialysate and observed for symptoms of infection. After 12 h of dwell time, residual dialysate was removed from the peritoneum and determined for viable planktonic cells. The rabbits received an additional 250 ml of dialysate containing a formulation and were observed for a further 12 h, then the experiment was terminated. Run 2 was modified such that 250 ml dialysate containing a formulation was administered only 12 h after implantation of a lifesaver device. The administration of dialysate and formulation was repeated every 12 h for 2 days; each time the residual dialysate was removed and viable cell counts determined.

Determination of viable bacterial cells - Each disk of the lifesaver device was washed with 2 ml of PBS to remove surface planktonic cells. Each side of the disk was scrapped with a sterile scalpel blade into 2 ml of sterile PBS, sonicated, serially diluted, aliquoted and plated on nutrient agar. After 18 h, the plates were counted to determine the colony-forming units per cm^2 of the disk (cfu/cm^2). Populations of planktonic cells per ml of residual dialysate (cfu/ml) were determined from serially-diluted samples incubated on nutrient agar plates.

7.2.4 Post Mortem Examination

Samples of the lifesaver device, the CAPD catheter and the peritoneal wall were collected from animals immediately after they died of the infection or following euthanasia. Bacterial counts were obtained from peritoneal fluids, lifesaver devices, interior and exterior walls of the CAPD catheter, and the peritoneal walls of all of the animals. Bacteria were enumerated as described above. The peritoneal wall

tissues associated with the implant device were fixed in neutral buffered formalin, sectioned and stained with haematoxilin and eosin according to standard procedures. Histological sections were examined with a light microscope by a veterinary pathologist. Scanning electron microscopy (SEM) was used to study the surface produced of one of the disks of each implanted lifesaver device and of the CAPD catheter following the procedure of Ward et al. (27).

7.3 RESULTS

The absorption of CFX from the peritoneum and its elimination from peritoneal dialysate as a function of time are shown in Figure 7.1, and the relevant pharmacokinetic parameters are given in Table 7.1. After 30 min, a C_{max} of 48 μg/ml was obtained in the peritoneal dialysate from the microspheres and this level was maintained for about an hour. Susbsequently, concentrations fell exponentially similar to that observed for the free drug. After 12 h the concentration of CFX was approximately 3 μg/ml for microspheres compared to 1 μg/ml from free drug.

The corresponding serum concentrations of CFX are shown in Figure 7.2 and the pharmacokinetic parameters are shown in Table 7.2. The serum concentration-time profile from the solution formulation typically displays an absorption phase, a C_{max} of 1.8 μg/ml which occured T_{max} = 1 h, and an elimination phase which after 12 h reduced the CFX concentration to almost zero. In comparison, serum concentrations derived from the microsphere formulation increased rapidly at first to about 1.2 μg/ml, then gradually increased to about 1.6 μg/ml after 4 h, a level

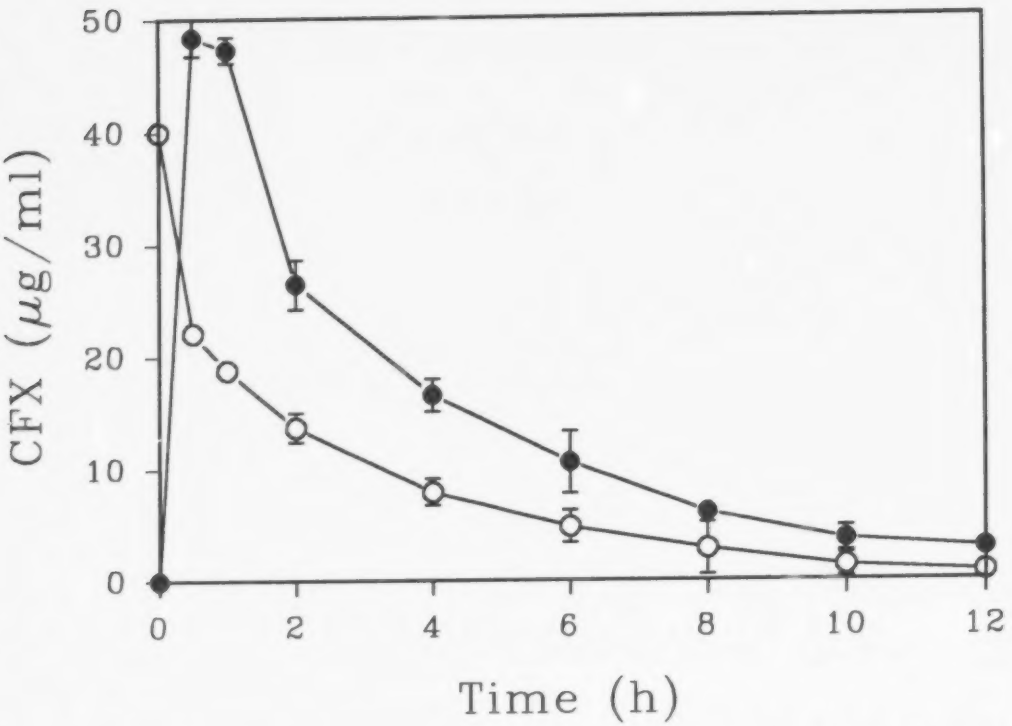

Figure 7.1 Mean dialysate concentration levels as a function of time for 2 groups of rabbits after a single intraperitoneal dose of: (O) 10 mg of free CFX, (●) 20 mg of CFX in PLA microspheres.

Table 7.1 Kinetics of CFX disappearance from the peritoneum of rabbits following administration of 20 mg of CFX in microspheres or 10 mg of free CFX in peritoneal dialysate

Formulation	C_{max}[1] (μg/ml)	T_{max}[2] (h)	C_{12h}[3] (μg/ml)
Free drug	40(0.0)	0.0(0.0)	1.0(0.3)
Microsphere	48(1.6)	0.5(0.0)	2.9(0.2)

[1]initial or maximum concentration attained in the peritoneal dialysate. Standard error of the mean (SEM) shown in brackets.

[2]time to reach C_{max}.

[3]concentration after 12 h.

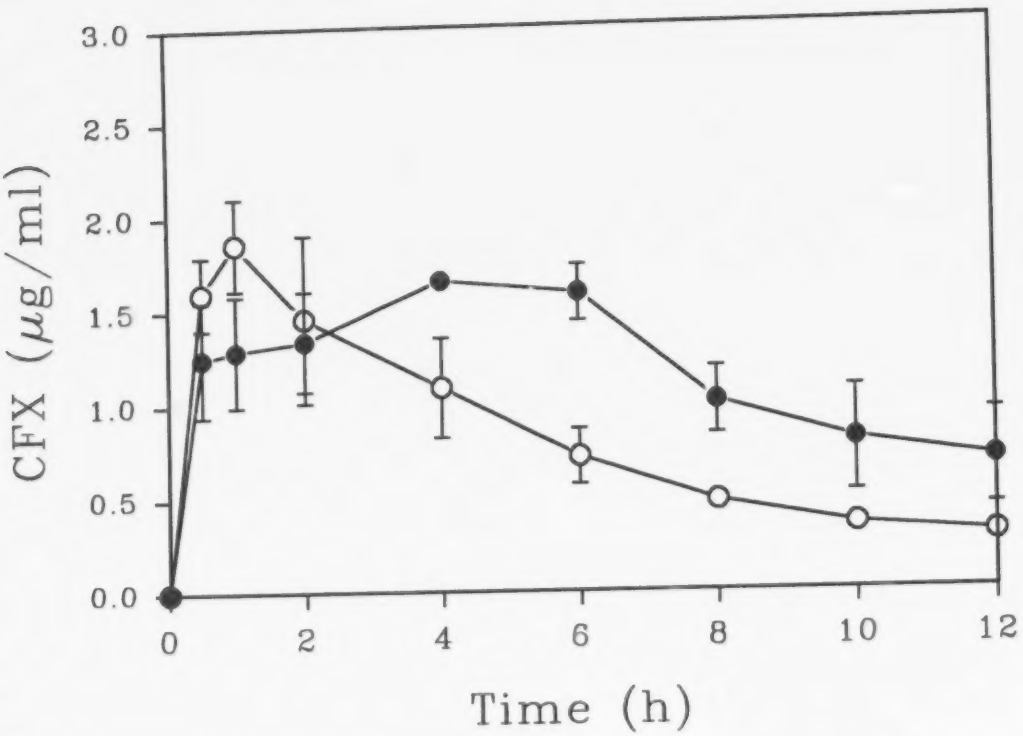

Figure 7.2 Mean serum concentration levels as a function of time for 2 groups of rabbits after a single intraperitoneal dose of: (O) 10 mg of free CFX, (●) 20 mg of CFX in microspheres.

Table 7.2 Kinetics of CFX appearance in serum of rabbits following administration of 20 mg of CFX in microspheres or 10 mg of free CFX in peritoneal dialysate

Product type	C_{max}[1] (μg/ml)	T_{max}[2] (h)	C_{12h}[3] (μg/ml)
Free drug	1.8(0.3)	1.0(0.0)	0.3(0.1)
Microsphere	1.6(0.1)	4.0(1.0)	0.7(0.3)

[1]maximum concentration in the serum. SEM shown in brackets.

[2]time to reach C_{max}.

[3]concentration after 12 h.

which appeared to be sustained for 2 h before decreasing to lower levels with a C_{12h} value of 0.7 μg/ml. The effect of the anaesthetic agent or the physiological state of the rabbit on metabolism of serum CFX was not investigated since we were mainly interested in peritoneal CFX levels.

The mean population of viable *P. aeruginosa* cells that colonized each lifesaver device prior to implantation was 9×10^7 cfu/cm^2. In the initial experiment (run 1), the mean population in the peritoneal dialysate had risen to 2×10^8 cfu/ml after 24 h. Also, rabbits displayed symptoms of septic shock 1-2 h after the beginning of the experiment and 2 out of 2 rabbits in group 1 (no antibiotic), 2 out of 2 rabbits in group 2 (free antibiotic), and 1 out of 2 rabbits in group 3 (CFX microspheres) died within 24 hours. Post-mortem examination indicated death was associated with bacterial endotoxemia and septicemia. The single surviving rabbit that had received CFX microspheres recovered fully. It was concluded that peritonitis in untreated animals was fatal and this group was excluded from run 2.

In the subsequent experiment (run 2), analysis of the residual peritoneal dialysate 12 hours after administration of antibiotic yielded a mean *P. aeruginosa* planktonic cell population of 1.1×10^4 cfu/ml in group 2 (free CFX) after 12 h in the dialysate whereas the residual dialysate taken from rabbits in group 3 (CFX microspheres) was free of viable planktonic cells (Table 7.3). After 24 h, two of the rabbits in group 2 died of toxemia and septicemia and the third rabbit was very sick whereas all three rabbits in group 3 survived and were clinically normal. After administration of CFX microspheres at 24 h and 36 h a low level of planktonic cells

Table 7.3 Population (mean±SEM) of viable planktonic cells determined in residual peritoneal dialysate 12 h after administration of free CFX (group 2) or CFX in microspheres (group 3) to rabbits

Time(h)	Group 2[1]	Group 3[2]
12	4.04(0.02)	NG
24	3.56(0.07)	1.56(0.49)
36	2.9	1.40(0.12)

NG, no growth. SEM, standard errror of the mean.

[1]Log cfu/ml in residual dialysate. Mean CFX concentration 12 h after each administration of free CFX was 1.1(0.3) μg/ml. Only 1 rabbit in this group survived after 36 h.

[2]Log cfu/ml in residual dialysate. Mean CFX concentration was 2.7(0.8) μg/ml.

were found in the residual dialysate (Table 7.3).

The population of viable bacterial cells per gram of peritoneal wall tissue was 1000 times larger in rabbits which received free CFX compared to rabbits that received CFX microspheres (group 2 versus group 3, Table 7.4). Likewise, the mean population of cells (cfu/cm) of interior or exterior wall material of the peritoneal catheter from group 2 rabbits was approximately a 1000 times that found in group 3 rabbits. One rabbit in group 3 contained no viable bacterial cells on the catheter or in the residual dialysate. Disks from the lifesaver device implanted in two of the three rabbits in group 3 contained 100-fold fewer viable cells than those in group 2 rabbits and disks removed from the third rabbit had no viable bacterial cells. Recovery of residual dialysate from 2 out of 3 rabbits in group 3 was incomplete, which resulted in lower CFX concentrations after subsequent administration of CFX microspheres in more peritoneal dialysate. This problem resulted in subpotent CFX concentrations to eradicate planktonic cells (Table 3) and biofilm cells on the devices (Table 4) in these two rabbits.

The scanning electron microscopy (Figures 7.3-7.8) and histology results (Figure 7.9 and 7.10) support the clinical and microbiology data. The peritoneal wall tissue recovered from group 2 rabbits was severely inflamed, edematous and necrotic. There was extensive infiltration of inflammatory cells (mostly neutrophils) and bacteria on the peritoneal surface. The tissue recovered from group 3 rabbits was only mildly inflammed, with moderate infiltration of inflammatory cells. The interior and exterior of the CAPD catheter was colonized with a thick bioflim

containing numerous bacteria (Figure 7.3 and 7.4). The biofilm was thicker with more bacteria on the inside of the catheter. A similar biofilm was observed on the disc of group 2 rabbits (Figure 7.5). In contrast, the interior and exterior of the CAPD catheters, and the discs from one rabbit in group 3 rabbits were free of biofilm and bacteria (Figures 7.6-7.8). Only the occasional red or white blood cell could be observed.

7.4 DISCUSSION

These studies have demonstrated unequivocally the health risk of rabbits infected with *P. aeruginosa* device-associated biofilm and viable planktonic cells if left untreated. After I.P. administration of a bolus dose of dialysate solution of CFX, the drug is rapidly cleared and enters the general circulation. Under the present conditions, a peak concentration of 1.8 μg/ml of CFX in serum occurred after 1 h then concentration decreased to 0.7 μg/ml after 6 h and 0.3 μg/ml after 12 h. As a result, rabbits which had received only free CFX succumbed to infection and subsequently died after about 12 h. The mortality of the rabbits could possibly have been reduced if frequent injections or infusion of antibiotic had been administered instead. Alternatively, sustained release microsphere formulation of CFX successfully treated the infection and prevented death. Although only one CFX microsphere formulation was tested, the results were remarkably positive. However, it could be argued that optimization of the treatment protocol would be attainable by modifying the formulation.

Table 7.4 Recovery of *P. aeruginosa* cells (mean log cfu±SEM) from Silastic implant, peritoneal wall and residual peritoneal dialysate of rabbits 24 h after the final administration of free CFX (group 2 = 1 rabbit) or CFX in microspheres (group 3 = 3 rabbits)

Rabbit group	Silastic implant[1]	Peritoneal catheter[2]		Peritoneal[3] wall	Peritoneal[4] fluid
		interior wall	exterior wall		
2	7.62(0.27)	5.08	4.78	9.08	6.58
3	5.52(0.20)	2.63(0.83)	2.65(0.83)	5.96(0.07)	3.15

[1]Log cfu/cm^2 of Silastic. Three disks per implant per rabbit.

[2]Log cfu/cm of interior and exterior walls.

[3]Log cfu/g.

[4]Log cfu/ml of residual amount. Dialysate was recovered from only one rabbit in group 3.

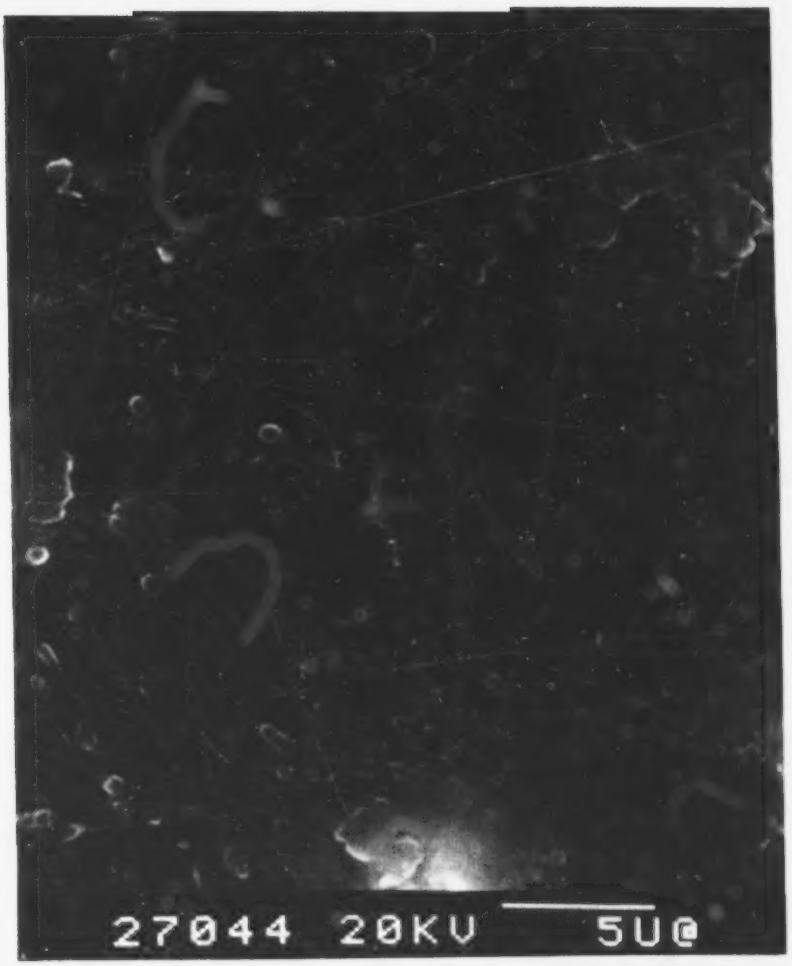

Figure 7.3 SEM of the interior of the CAPD catheter in group 2 rabbits. Note the thick biofilm and numerous *Pseudomonas aerginosa* cells embedded within the biofilm (the bar shows a line of 5 micrometres).

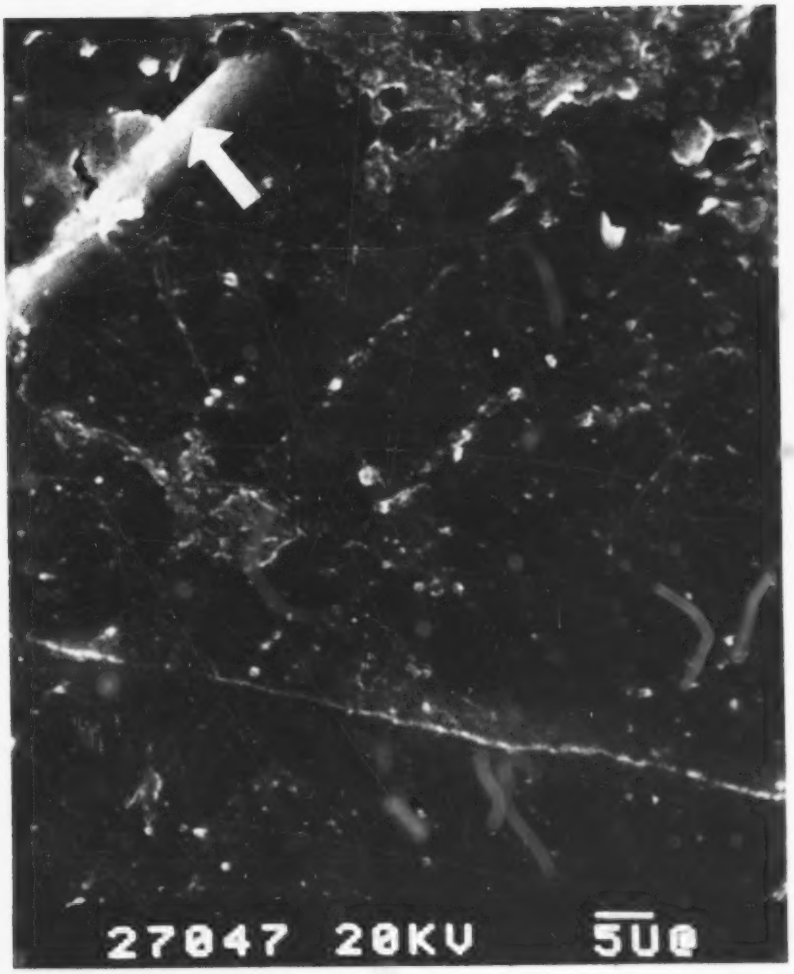

Figure 7.4 SEM of the exterior of the CAPD catheter of group 2 rabbits. Note the thinner biofilm and the exposed catheter surface (indicated by arrow).

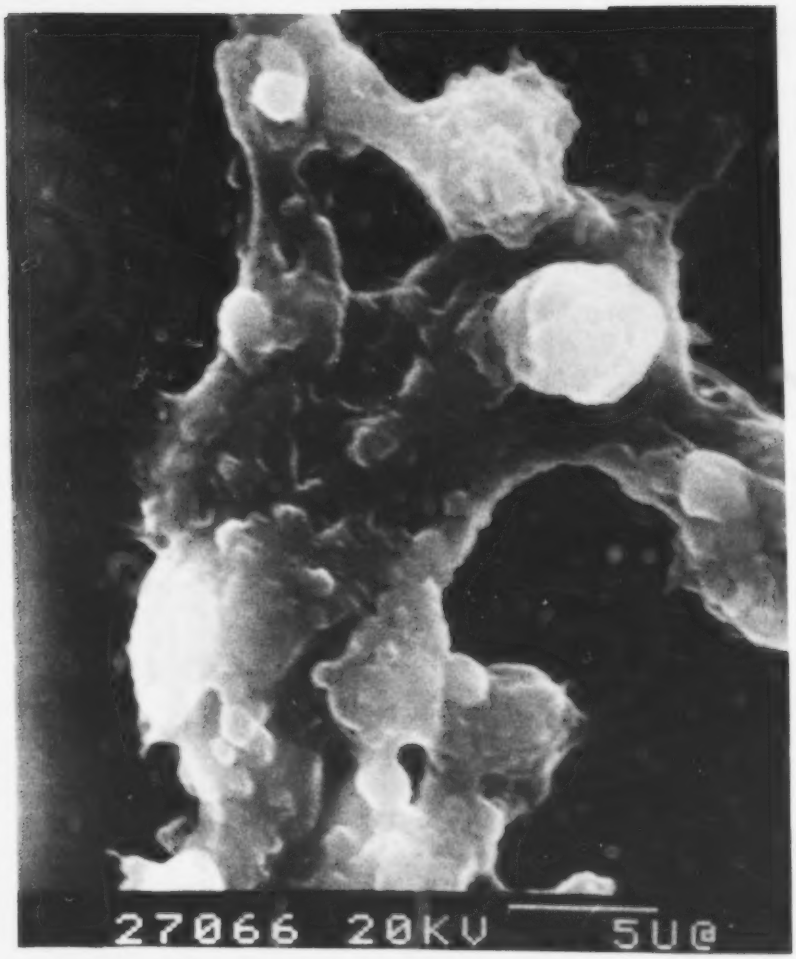

Figure 7.5 SEM of a disc of the lifesaver device in a group 2 rabbit. Thick biofilm containing bacteria on the surface of these discs can be seen.

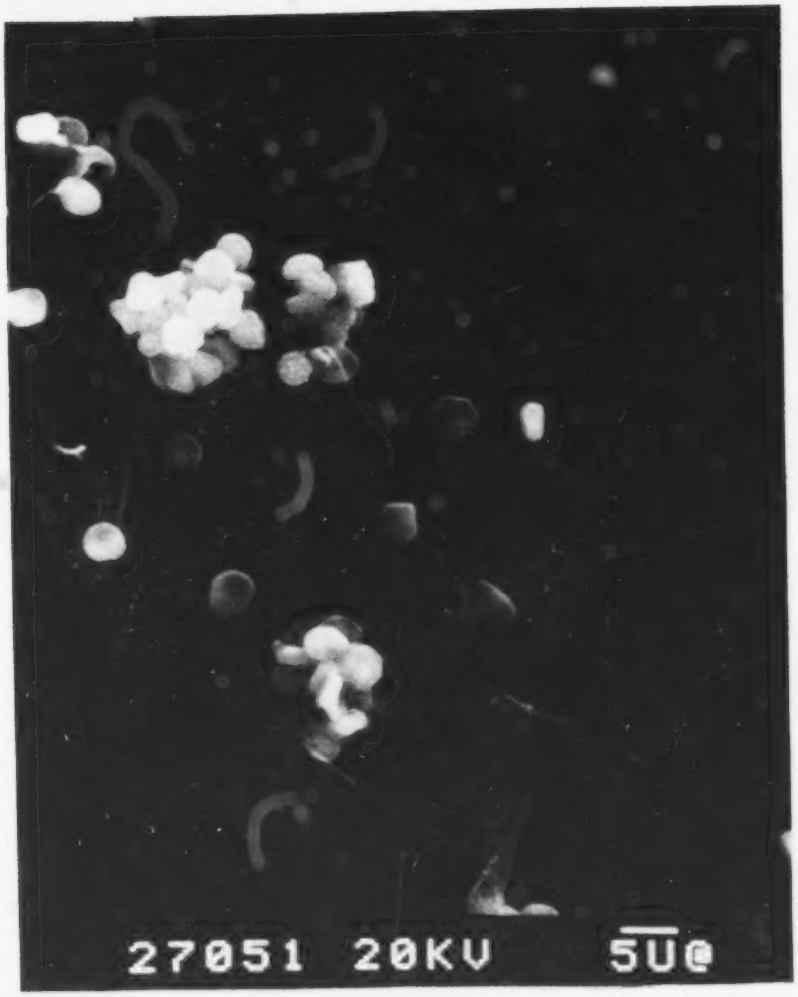

Figure 7.6 SEM of the interior of the CAPD catheter of group 3 rabbits. Note the absence of a biofilm and only the occasional erythrocyte.

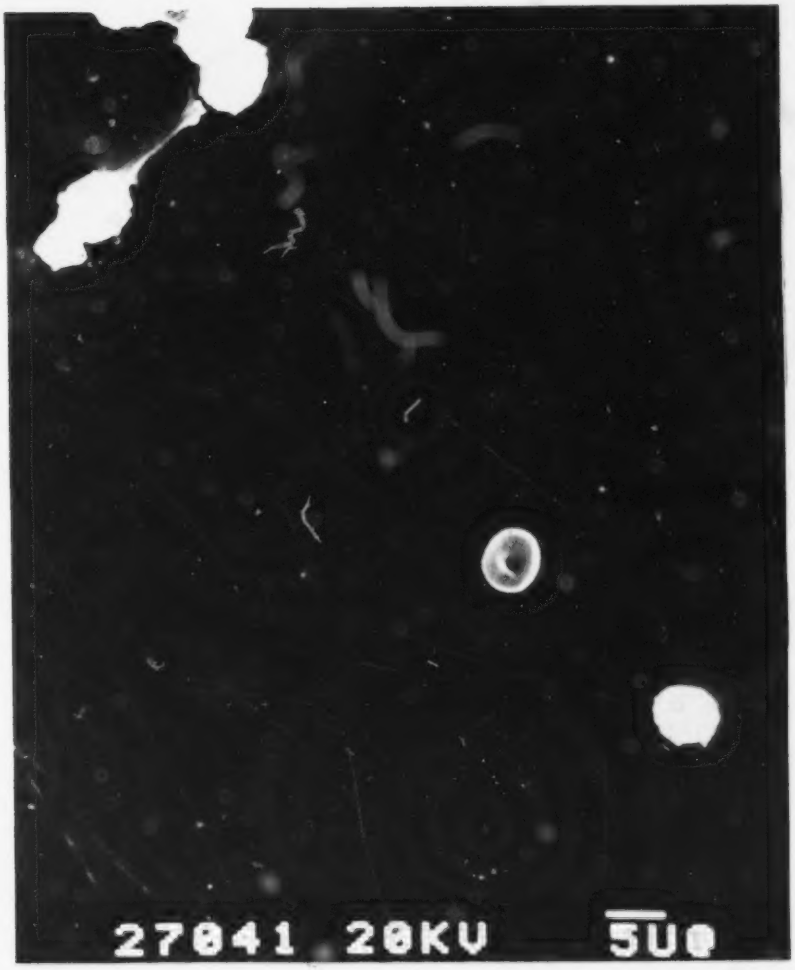

Figure 7.7 SEM of the exterior of the CAPD catheter of group 3 rabbits. Note the absence of a biofilm and only the occasional erythrocyte.

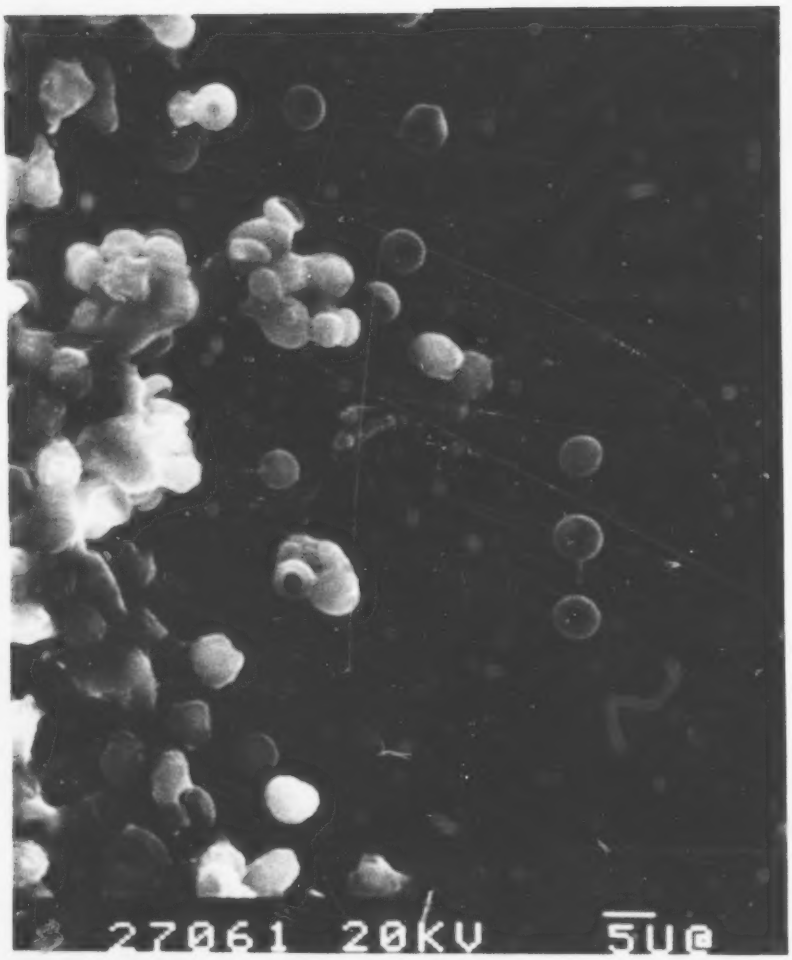

Figure 7.8 SEM of the disc recovered from a group 3 rabbit. Note the absence of the biofilm.

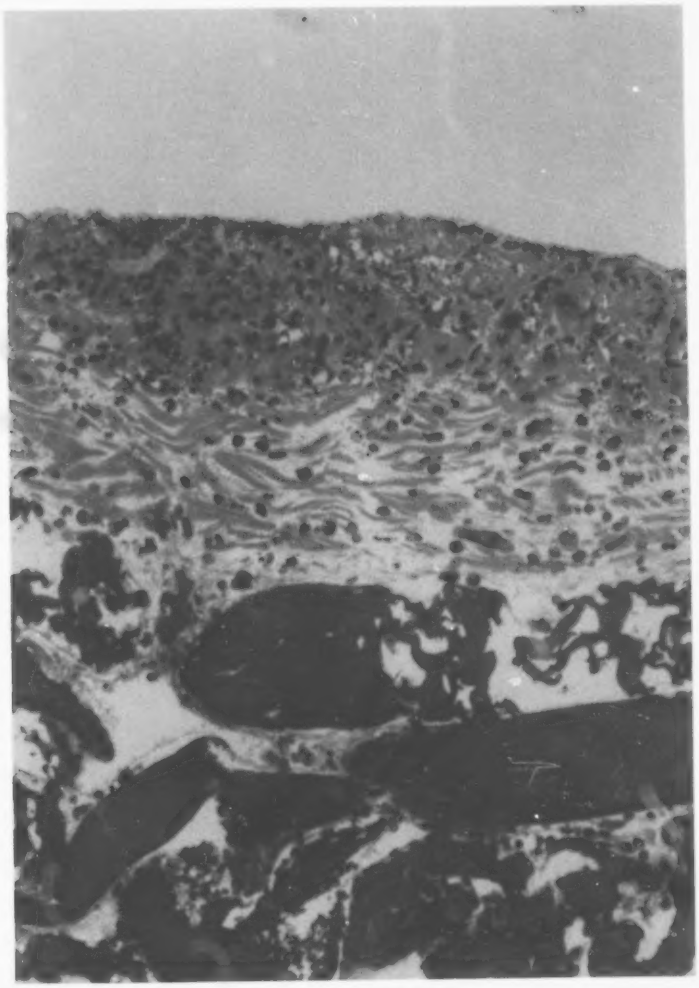

Figure 7.9 Histology of peritoneal wall tissue recovered from group 2 rabbits. Note the thicker fibrous connective tissue (upper layer) and severely inflammed muscle (lower layer) (x 20 magnification).

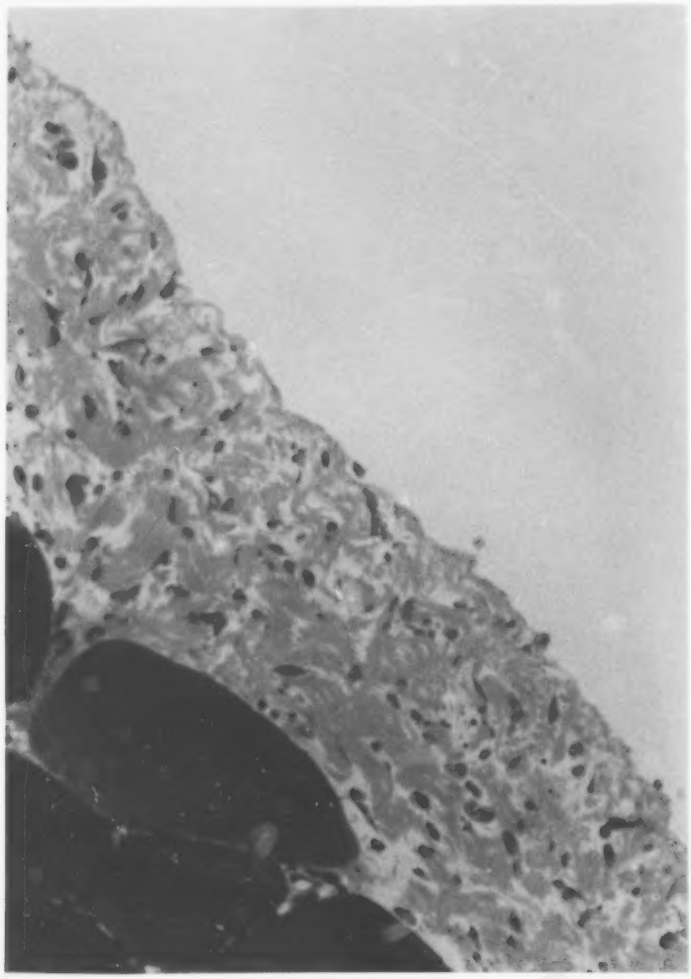

Figure 7.10 Histology of peritoneal wall tissue recovered from group 3 rabbits. The fibrous connective tissue (upper layer) and muscle (lower layer) are normal (x 20 magnification).

Previously, eradication of *P. aeruginosa* biofilm and planktonic cells in an open chemostat system had been shown using this CFX microsphere formulation.The kinetics of dilution of CFX in a constant volume of solution in the chemostat were comparable to the kinetics of CFX absorption from the peritoneum of the rabbit. Successful eradication of *P. aeruginosa* biofilm and planktonic cells in the open chemostat using sustained release CFX microspheres suggest that the formulation should behave similarly in the rabbit model. In both models (*in vitro* and *in vivo*) it has been shown that eradication of biofilm was possible when effective CFX concentrations were attained and then maintained without wide fluctuations. In terms of treatment of device-associated bacterial infection, only when the biofilm had been eradicated did the rabbit survive, indeed recovered completely. The depleted PLA microspheres, although being biodegradable, for the most part were removed with the residual dialysate. Thus there was no unnecessary accumulation of the polymer in the peritoneum. This contrasts with the fate of rabbits that received only free CFX, and with the unseccessful eradication of biofilm in the open chemostat system. This suggests that the open chemostat system is a good *in vitro* model for testing formulations subjected to pharmacokinetic processes at the peritoneal site of administration, especially, under conditions of localized infection. Therefore, use of the open chemostat for preliminary evaluation of dosage forms may enable reduction in the number of animal experiments that need to be carried out.

It can be predicted from the results of this experiment that sustained release

microparticles of antibiotic will be more efficient than free drug at equivalent maximum concentrations in treating device associated biofilm infections in humans on CAPD. The biofilm growing mainly in the interior of peritoneal catheter has been thought to be responsible for recurrent peritonitis in CAPD patients. The failure of many treatment modalities with free antibiotic in solution is due to ineffective antibiotic level in the vicinity of bacteria, especially those growing in biofilm. It has already been demonstrated in the shake flask or test tube which are closed systems that bacteria in biofilm require sustained high concentration of antibiotic for their eradication (9-11). This can be easily achieved in the test tube but not *in vivo* where drug is continuously cleared from the site of infection. In device related infections of CAPD patients, it is often difficult to eradicate all of the bacteria due to diminishing concentration of antibiotic after administration of initial high concentration. When treatment is stopped, the bacteria in biofilm multiply very fast and cause re-infection. On the other hand, administration of sustained release biodegradable polymeric microparticles will be able to deliver sustained levels of a killing dose of antibiotic to the vicinity of biofilm. Thus, the eradication of bacteria in biofilm will result in the cure of infections associated with the biofilm. This has been demonstrated in the peritoneum of rabbits. The thick biofilms observed in the rabbits which received free drug (Figures 7.3, 7.4 and 7.5) and their subsequent death unlike those which received microspheres indicate the beneficial effect that can be obtained from administration of sustained release formulations. The absence of biofilm on the devices recovered from rabbits which received CFX in

microspheres (Figures 7.6, 7.7 and 7.8) coupled with their high survival rate reinforces the need to consider sustained release biodegradable polymeric microparticulate formulation as an alternative to the current practice in the clinic, especially, where recurrence of peritonitis is diagnosed. In addition, systemic levels of the antibiotic which is responsible for its toxicity can be reduced by formulating the antibiotic as a sustained release product.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 GENERAL DISCUSSION

The formulation of antibiotics in biodegradable polymeric microparticles provides a convenient means for sustained release. The ideal sustained release period is dependent on the intended application of the product. In patients undergoing CAPD who become exposed to biofilm infections, the dwell time of the peritoneal dialysate imposes a time limitation on the functionality of the formulation. Thus, for a maximum dwell time of peritoneal dialysate of 8 h microparticles would be required to expend their antibiotic content during this period. Also, the rate of release should be sufficient to offset the rapid transport of antibiotic from the peritoneum to the systemic circulation, especially in peritonitis (1), and yield a concentration in the peritoneum high enough to be effective. The polymer and the process used to prepare the microparticles are paramount in achieving this objective. This study has shown that the coacervation conditions used to microencapsulate CPX or CFX with PLA produced microspheres which operated within this context.

Several techniques are available for the formulation of drugs in polymeric sustained drug delivery systems, however, the method chosen depends on the desired rate and kinetics of release, as well as the mode and frequency of administration. Steroids have been incorporated in polylactic acid by cast-molding and implanted in animals to release the drug over a duration of 6 months (2). This ensured slow release of the drug for a prolonged period of time. This approach, however, would not be suitable for the formulation of antibiotics for the treatment

of biofilm infections since high concentrations of antibiotic are required.

It has been reported that many microparticles form clusters during their preparation, particularly those produced by various coacervation procedures such as gelatin and ethylcellulose microcapsules (3). The shapes of the microparticles produced range from highly irregular to nearly spherical. CFX or CPX microspheres prepared with PLA 50,000 or 100,000 M.W. were nearly spherical whereas CFX microspheres prepared with PLA 300,000 M.W were irregularly shaped (Chapter 4). The high rate of precipitation together with a very high crystallinity are pressumed to be responsible for this. Although PLA was used for the preparation because of its well- established biodegradable and histocompatible properties, other polymers of the same family such as poly(DL-lactic acid) and copolymers of lactic/glycolic acids which degrade at a faster rate could be used (4,5)

The efficiency of microencapsulation is affected by the method of preparation. Proper selection of the solvent system is crucial to the quality as well as the encapsulation efficiency of the finished product. Microencapsulation of water-soluble drug particles by phase separation requires the use of vehicles which are poor solvents for the drug in order to achieve high efficiency of encapsulation. This criterion was suitably met for CPX and CFX using methylene chloride and diethyl ether as the polymer solvent and nonsolvent, respectively. As a result, encapsulation efficiencies were very high, being >90% with minimum batch-to-batch-variability. Such results might not have been achieved if a 2-phase-system

(eg. an o/w emulsion) had been used where part of the drug or polymer would likely transfer to the the continuous phase and be lost.

Of all the techniques available for the formulation of microparticles, coacervation technique is the one that results in the formation of porous products (3,6). Microencapsulation of water-soluble drugs by coacervation using nonsolvent addition procedure has resulted in the formation of porous microparticles which made drug available at a much slower rate than that obtained from dissolution of the drug but not as slow as that which would be obtained if the drug particles were surrounded by a continuous polymer membrane or matrix. In the case of CPX or CFX an initial high concentration of antibiotic was sustained depending on the microsphere size, polymer composition and processing conditions (Chapter 3 and 4). SEM pictures provided evidence of the porous nature of microspheres produced during preparation. Although it was expected that microspheres would be produced where kinetics of release would be governed by diffusion through a polymer phase, microspheres of CFX or CPX prepared by a coacervation procedure resulted in a release rate driven by the rate of dissolution due to incompletely coated drug particles in the matrix. The second order dissolution kinetic model, not the first order, cube root law, or Weibull distribution theory, gave best fit of the data which is interpreted to mean that the effective surface area exposed to the release medium and the weight of the drug control the kinetics of release. Polylactic acid microparticles of other drugs have been reported to obey matrix release kinetics after an initial burst phase (7,8). It is important to note that

rate of release from microspheres prepared by this procedure would be much slower if release were governed by diffusion of drug through monolithic polymers (2,9).

In general, the kinetics of release from microparticles have been characterized by zero order, first order, matrix or dissolution models (3). Although the profiles of release in a closed system give useful information about the mechanism of drug release, this situation is not realistic *in vivo* where drug is continuously being removed from the site of administration and the body. Closed system data are mainly useful in helping to characterize formulations but not elucidating how they will behave *in vivo*. Moreover, correlation of closed system release data with expected release profiles *in vivo* is often not good. The application of the open chemostat system to study drug release from microparticles where drug can be continuously removed from the system by adjusting the system dynamics offers an opportunity to simulate *in vivo* concentration profiles (Chapter 5). In the first instance, knowledge of elimination kinetics of the drug in the body can be very useful in selecting the appropriate dilution rate in the open chemostat system. The chemostat system is especially useful for studying the release of drug from delivery devices that are intended for localized drug delivery (e.g. in the peritoneum), so as to simulate drug concentration time profiles at the site of administration.

The open chemostat system does not appear to have the problem of clogging and pressure build-up associated with some flow through systems (10,11).

Microparticles are sealed in a polyethylene screen to retain them in the chemostat and contents of the chemostat are aerated at a constant air-flow rate unlike other flow through systems. Although the chemostat system cannot be used to replace animal experiments, it has been demonstrated that it can be applied to screen sustained release microparticulate formulations. This might hopefully reduce the number of animal experiments that need to be carried out prior to human testing.

There is increasing evidence that clinicians and researchers are now paying more attention to biofilm-related problems of implanted devices. This is not surprising since the application of medical devices in modern medical care has been rapidly increasing. This was not the case in the past where a number of scientists discounted the significance of biofilm as being responsible for re-infection and mortality of several patients using medical devices. Recently, the incorporation of antibiotics or anti-inflammatory agents in silicone tubing material or the search for implant materials that prevent adherence of microbes to medical devices have been attempts to solve the biofilm problem (12,13). However, these have not met with much success because tubing becomes exhausted of antibiotic over a period of time. Also of interest would be agents that would have selective toxicity to the exopolysaccharide matrix of biofilm in order to expose the biofilm cells thereby restoring sensitivity to usual concentrations of antibiotics. Although bacteria in biofilm are more resistant to antibiotic than planktonic cells, Anwar et al. (14) demonstrated that exposure of biofilm to a high concentration of antibiotic in a test

tube was effective in eradicating these cells. But in a closed system, such as the shake-flask or test tube, bacteria in biofilm can be easily exposed to a fixed concentration of antibiotic solution whereas *in vivo* attainment of an effective concentration of antibiotic is not straight-forward because of drug transport, distribution and elimination processes which cause a fairly rapid lowering of antibiotic concentrations. This means that effective antibiotic concentrations are not maintained adjacent to the site of infection for a sufficiently long period of time, which is required to eradicate biofilm on the device. This principle has been successfully demonstrated by *in vitro* simulation of antibiotic concentrations in the open chemostat model where diminishing concentrations of antibiotic resulted in a concentration which was ineffective to eradicate bacterial biofilm (Chapter 6).

The successful application of CFX microspheres to treat aged biofilm in the open chemostat system under a condition mimicking the *in vivo* situation (Chapter 6), signalled the need for further testing in animals. Anwar et al. (15), and Nickel et al. (16), observed that the concentrations of antimicrobial agents required for the eradication of bacteria in biofilms are over ten-fold higher than those needed to kill planktonic cells. The complex nature of the physiology of biofilm cells and the thick glycocalyx which they produce protect them against antibiotic action and as such are different from those grown planktonically. Only by using a sustained release drug delivery device could such a high concentration be maintained *in vivo* or in the open chemostat. In the context of these studies, a 71% w/w PLA (100,000 M.W.) microsphere formulation in the 250-425 μm sieve size range provided the

required drug concentration to eradicate biofilm bacteria of *P. aeruginosa* and *S. aureus*, whereas those of lower drug loadings were ineffective at the same dose level. This information obtained from the chemostat proved to be applicable *in vivo* in the rabbit peritoneum model.

The rabbit peritoneal model of biofilm growing on a lifesaver device was particularly useful for comparing the relative effectiveness of CFX in solution and in the microsphere formulation (Chapter 7). It was possible to demonstrate both in the open chemostat and in the rabbit peritoneal model, that sustained release microparticles were more effective in eradicating bacteria biofilm than free drug at equivalent maximum concentrations. By sustaining drug concentrations both in the peritoneum and serum, microspheres administered with the peritoneal dialysate maintained drug concentrations sufficient to eradicate device-associated biofilm infections in the rabbit. This finding makes it possible to predict that microparticles of the type used here have the potential for treating recurrent peritonitis in CAPD patients, by administering the microparticles through the peritoneal catheter to achieve localized drug delivery. Hopefully, this approach will reduce the incidence of recurring episodes which currently stands at 10-30% (17) due to treatment failure following administration of dissolved antibiotic in peritoneal dialysate solution (1,18). This should lead to a reduction in the mortality rate (2.4-12.2%) due to peritonitis in CAPD patients which represents 17.2-50% of all deaths (19). Furthermore, treatment interruption due to removal of the catheter and placement

of the patient on hemodialysis will be reduced. This will go a long way to increase the acceptance of the CAPD technique, and ultimately improve upon the quality of life of patients with end-stage renal disease.

Future studies would involve the testing of PLA microspheres in humans undergoing CAPD and to compare this treatment modality with the current practice in the clinic. Other biodegradable polymers such as poly(DL-lactic acid) or copolymers of lactic/glycolic acids as well as polyanhydrides, polyisobutylcyanoacrylate and poly(caprolactone) should be examined for similar improvements using smaller sized microparticles.

8.2 CONCLUSIONS

1. The rate of killing of *P. aeruginosa* bacteria in the open chemostat system under conditions of changing concentrations of CFX was found to be higher for planktonic than for biofilm cells. Bacteria in biofilm are therefore able to protect themselves against antibiotic action.
2. Using the phase separation procedure by nonsolvent addition of preparing microspheres of cephalexin or ciprofloxacin, the encapsulation efficiencies were consistently >90%.
3. During processing of microspheres, a stirring rate of 400 rpm yielded a minimum second order release rate constant, k_2, and the most uniform microsphere characteristics.
4. The release of CPX or CFX from microspheres obeyed second order kinetics, and SEM pictures indicate the porous nature of microspheres prepared. The release rate observed in the case of CPX microspheres was higher compared to CFX microspheres of the same size and composition, due to about a ten-fold difference in solubility.
5. An increase in drug loading of CPX microspheres from 23% to 33% w/w resulted in a decrease in release rate but the trend reversed with further increase in loading. Since the rate of release was a dissolution controlled process, the optimum efficiency of coacervate deposition relative to the surface area exposed by the drug particles was obtained at 33% w/w loading.

6. The rate of release of CPX or CFX could be controlled by varying the microsphere size, drug loading, molecular weight of PLA, or using a PLA/PGA composition.
7. The release of CFX from PLA microspheres was sustained for twice as long as CPX from equivalent formulations.
8. Peak concentrations of released antibiotics in the open chemostat obtained from microspheres were lower and more extended compared to free drug, but CPX profiles from microspheres were of shorter duration compared to CFX of the same composition and size.
9. The open chemostat system was particularly versatile for studying the kinetics of dissolution, and release as a function of formulation parameters, with, or without pieces of silicone tubing containing biofilm.
10. The release profiles of microspheres could be adjusted in the open chemostat to mimic drug concentration-time profiles found *in vivo* after intraperitoneal administration. This suggests that the use of the open chemostat system in formulating and testing new sustained release microparticulate preparations could possibly reduce the need to use animals.
11. In the testing of CFX against biofilm bacteria growing on an implant material, daily administration of 71% w/w CFX microspheres provided adequate concentration to kill bacteria in 3 days while those of 50 or 33% w/w were ineffective at the same dose level. A 50% increase in the dose reduced the effective bacteria kill time from 3 to 2.5 days.

12. In the chemostat containing biofilm, C_{max}, T_{max}, and C_{24h}, increased with an increase in drug loading.
13. Sustained release of CFX from microspheres in the open chemostat was shown to be effective in killing bacterial biofilms whereas equivalent maximum concentrations of free drug were ineffective.
14. In the testing of CFX against *P. aeruginosa* biofilm in the rabbit peritoneal model, the dialysate and serum concentrations after 12 hours were greater using microspheres than free drug, although maximum serum concentrations C_{max} (serum) was lower and T_{max} was longer.
15. Rabbits which received only free drug died from implanted biofilm infections whereas those which received CFX microspheres recovered. Examination of SEMs of the catheter surface and histology of the peritoneal wall revealed that this was due to virtual elimination of *P. aeruginosa* which was not the case after free drug administration.
16. The application of microparticles of antibiotic to treat biofilm infections associated with peritoneal implants appears to be more effective due to sustained levels of antibiotic compared to the administration of free drug in peritoneal dialysate at equivalent maximum concentrations. It is expected that sustained release microparticles of antibiotic if administered to patients undergoing CAPD, could reduce the incidence of recurrent peritonitis, thereby reducing mortality rates and improving acceptance of CAPD procedures.

8.3 REFERENCES

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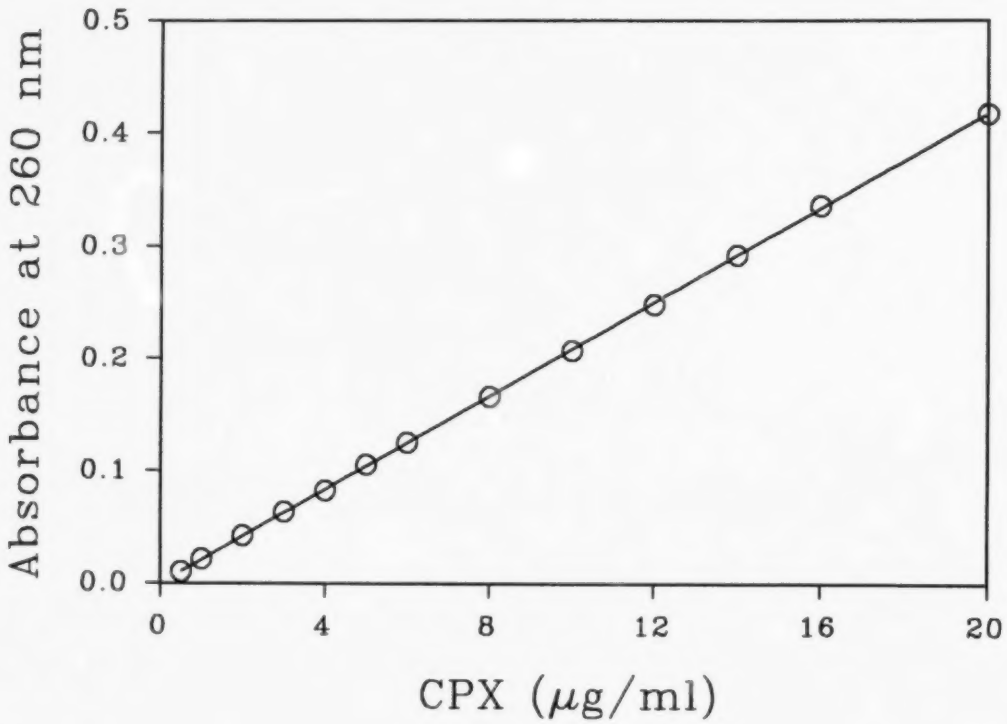
Appendix 1

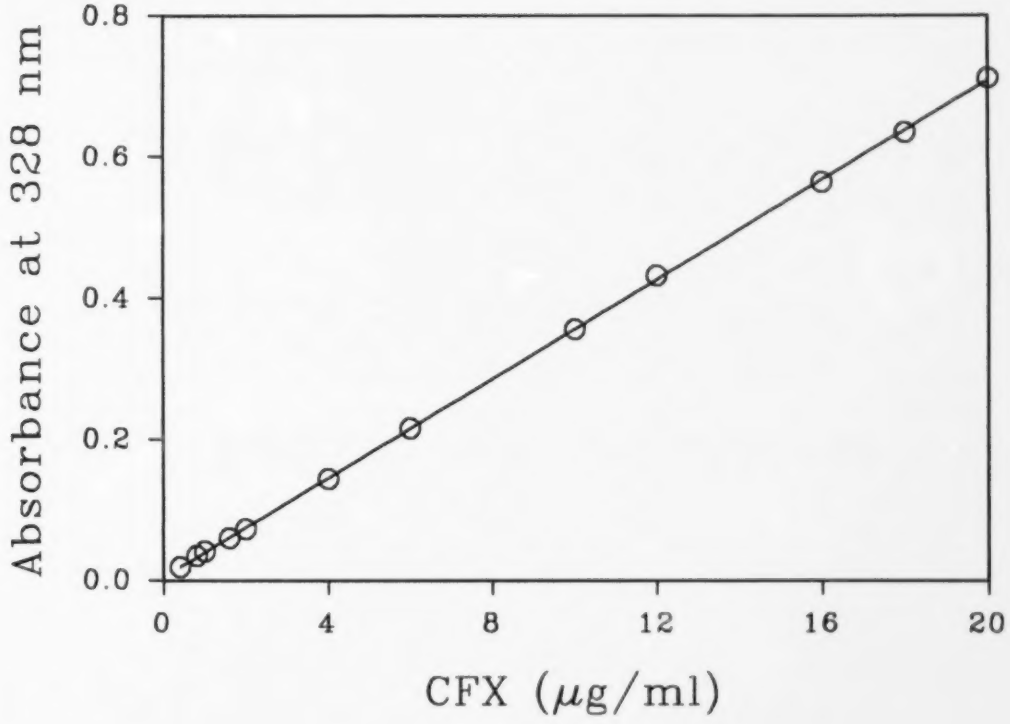
A) UV Spectrophotometric Analysis of Cephalexin

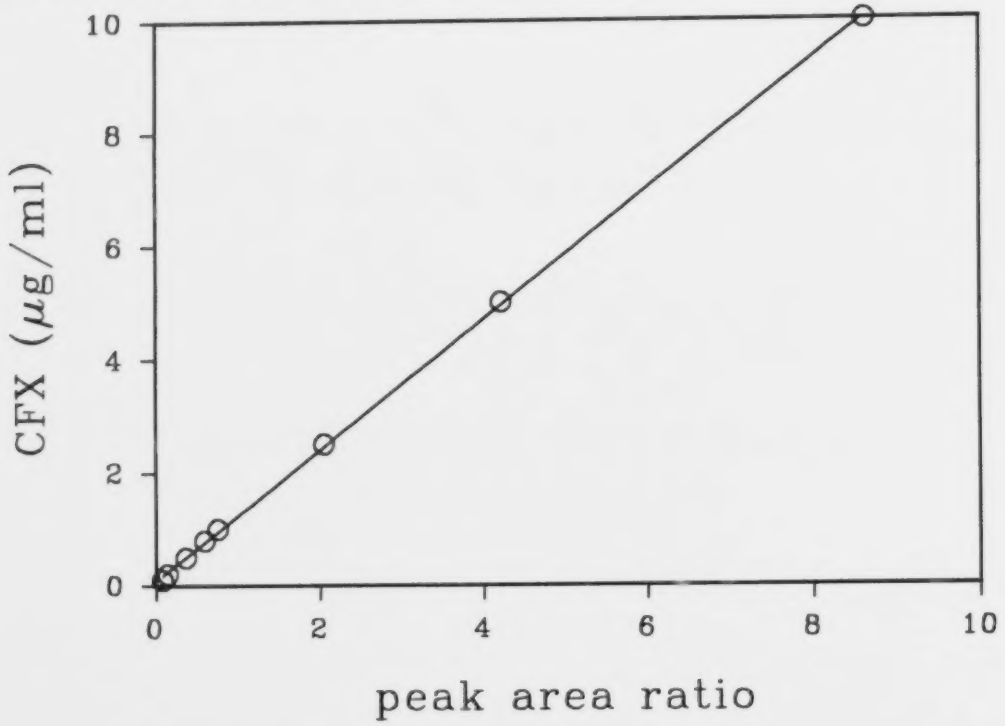
A linear calibration curve which obeyed Beer's law over the concentration range of 0.5 to 20 µg/ml was obtained for cephalexin as shown below. Regression analysis of the experimental values yielded a slope = 0.0209, intercept = -0.0003, and correlation coefficient, r = 0.9999. Reproducibility of the experimental values was always >99%.



B) UV Spectrophotometric Analysis of Ciprofloxacin

A linear calibration curve which obeyed Beer's law over the concentration range of 0.4 to 20 µg/ml was obtained for ciprofloxacin as shown below. Regression analysis of the experimental values yielded a slope = 0.0352, intercept = 0.0037, and correlation coefficient, r = 0.9999. Reproducibility of the experimental values was always >99%.



C) Fluorescence Analysis of Ciprofloxacin

A linear calibration curve which obeyed concentration range of 0.1 to 10 µg/ml was obtained for ciprofloxacin as shown below. Analysis was carried out by reverse phase HPLC with quinine sulphate as the internal standard. Excitation and emission wavelengths were set at 280 and 455 nm, respectively. Regression analysis of the experimental values yielded a slope = 1.154, intercept = 0.0879, and correlation coefficient, r = 0.9999. The daily coefficient of variation was <4%.